Pathogenicity of alpha-synuclein in various cell models for Parkinson's disease

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CHAPTER 1

GENERAL INTRODUCTION

Demographic changes and neurodegenerative diseases

According to the World Health Organization (WHO) (2015), the world is aging as illustrated in the graph in Figure1. The aged population is larger in the high-income Western countries than for instance in Mexico, Russia, South-Africa and Brazil. However, Brazil also already experiences changes in its demographic profile with a significant increase of aged population (IBGE). Technological advances allow the development of new therapies, leading to an increased life expectancy. Consequently, the percentage of aged people older than 60 years is growing in the entire world. From 2015 to 2050, an increase of 22% in the aged world population is expected. That would lead to an increased burden of age-related health problems to the society, demanding it to adapt their health systems.

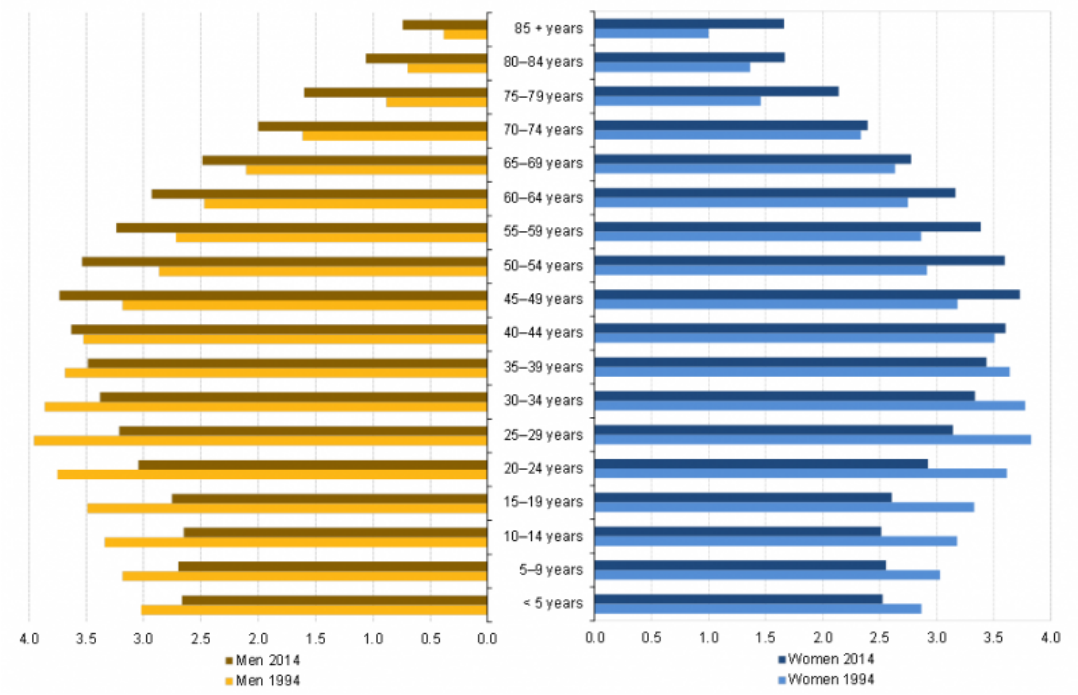

Figure 1. Illustration of European demographic changes from 1994 to 2014. Population of adults and aged men and women increased significantly from 1994 to 2014. Eurostat Statistic explained.

One of the most prominent age-related health problems is dementia, a broad category of brain diseases, characterized by a chronic and progressive loss of memory and a decrease in social behavior and cognition abilities. Nowadays, 47.5 million people

worldwide have dementia. It is expected that in 2050 there will be 135.5 million people living with dementia, mainly in low and middle-income countries such as Brazil and other countries in Latin America, where the prevalence of dementia is the highest compared to other regions in the world (Prince et al., 2013; Fagundes et al., 2011).

Dementia not only has an impact on people's life but also on their families and society since it provides a major burden as far as care and costs are concerned. Until 2010, health systems around the world spent around US$ 604 billion per year on the treatment of dementia. The most common disease that leads to dementia is Alzheimer's disease (about 60-70% of cases of dementia is related to Alzheimer's disease), followed by diseases like vascular dementia, dementia with Lewy bodies and Parkinson's disease (PD). Yoritaka and collaborators (2016) have shown that the direct costs for PD outpatient clinics per month, at the University Hospital in Japan, are USD 485.74 per subject. Furthermore, 90.6% of the costs are related to drugs to treat PD. They found that disease severity did not increase medical costs and they claimed that the costs in Japan are similar to Western countries.

Parkinson's disease development

PD was described for the first time by James Parkinson in 1817 (Parkinson 1817; for a review see (Goetz, 2011), and it is considered to be the most common neurodegenerative movement disorder (Tanner, 1992). It is estimated that PD affects 2% of the population older than 60 years old. According to the Parkinson's Disease Foundation (2016), men are 1.5 times more affected by PD than women and there are approximately 10 million people affected by PD around the world. Clinical symptoms of PD involve motor dysfunction such as muscle rigidity, bradykinesia, balance disturbances, resting tremor, and non-motor symptoms such as cognitive decline, depression and deficit in olfactory and gustatory systems in the early stages of the disease; the late stages of the disease include mood alterations, sleep disturbances and dementia (Cecchini et al., 2015; Chao et al., 2015; Paillusson et al., 2016; Saito et al., 2016; Weintraub et al., 2008).

Aging seems to be the main risk to develop PD. Degeneration of the substantia nigra (SN) is the main pathologic hallmark of PD and, therefore, it has been extensively

investigated. The degeneration of the dopaminergic neurons located in the compact part of the SN has been suggested to start in the distal axon retrogradely disturbing and inhibiting fast axonal transport. The dopaminergic phenotype slowly disappears as evidenced by the decreased levels of dopaminergic markers such as tyrosine hydroxylase (TH). As a compensatory reaction, the expression levels of dopamine receptors such as D1 and D2 have been found to increase. Interestingly, these changes have also been observed during normal healthy aging (Keeler et al., 2016; Rangel-Barajas et al., 2015; Thanos et al., 2016).

The first studies on PD pathology and aging showed a depletion of neurons as well as a decrease of pigmentation in the SN in both PD patients and healthy elderly subjects. The SN appears as a black structure in post-mortem brain tissue due to the cellular presence of neuromelanin, which is a pigment that accumulates throughout life at this region (Cabello et al., 2002; Rudow et al., 2008; Zucca et al., 2017).

As a consequence of the loss of the nigrostriatal dopaminergic projections, the levels of dopamine gradually decrease in the striatum during PD progress. Curiously, striatal dopamine levels also seem to decrease in aged healthy brains in a range about 10-13% per decade of life, and denervation of striatum is also found in the aged healthy brain (Carlsson and Winblad, 1976; Haycock et al., 2003; Hornykiewicz, 1989; Kish et al., 1988; Kish et al., 1992; Riederer and Wuketich, 1976). Some researchers suggested that an increased dopamine-turnover might be a compensatory mechanism in the degeneration of dopaminergic neurons in PD. Interestingly, a similar compensatory mechanism was observed by other researchers investigating aging of the SN (Barrio et al., 1990; Greenwood et al., 1991; Sossi et al., 2002).

Disturbed dopamine metabolism may increase intracellular oxidative stress. Although dopamine levels are decreased during aging and PD, oxidative stress is present in both situations. This changed redox state in dopaminergic neurons is thought to be caused by mitochondria dysfunction. It is known that oxidative stress can lead to progressive accumulation of oxidative damage, which is known to accelerate aging and PD development (Jang and Van Remmen, 2009; Kuter et al., 2016; Zucca et al., 2017).

It is important to point out that a major factor in the process of human and animal aging involves heritability of longevity, suggesting that the aging process is not

only modulated by life-style, but also has an important genetic component. Studies focusing on genes associated with the vulnerability of dopaminergic neurons have shown that genes involved in dopaminergic degeneration are also associated with normal aging. The most studied genes associated with PD appeared to be involved in the quality control of mitochondria and the modulation of oxidative stress; the same genes are associated with aging acceleration. α-Synuclein is an important pre-synaptic protein that plays a role in all neurons in the recycling of vesicles in synapses. Overexpression or anomalous conformation of this protein due to the presence of point mutations as well as a high oxidative environment lead to the oligomerization of α-synuclein and the formation of amyloidogenic filaments. This will lead to the formation of aggregates and Lewy bodies, which are found in both PD and healthy normal brains (Devi et al., 2008; Giasson et al., 2000; Li et al., 2004; Passarino et al., 2016; Polito et al., 2016; Prinzinger, 2005; Weihofen et al., 2009; Yang et al., 2016).

Various researchers are investigating the differences between aging and PD in the brain. PD differs from aging mainly with respect to the higher level of cell loss (Rodriguez et al., 2015). It seems that PD is a consequence of aging restricted to a specific cell population in the brain, whereas aging itself affects all cells in the body. Moreover, only 4-5% of aged people develop PD. With so many similarities between aging and PD development, it is hard to conclude what changes lead to the disease. PD is thought to be the consequence of a complicated interaction between a wide variety of potentially toxic external stimuli and variable genetic susceptibility explaining the high clinical diversity among PD patients.

Therefore, together these changes observed in aged brain could be similar in the PD brains, leaving unclear the threshold between healthy aging and neurodegeneration.

Risk factors for Parkinson's disease

The etiology of PD is still largely unknown. Among the PD cases, 95% are considered sporadic and only 5% has a known genetic cause. Manifestation of PD has been thought to involve the chronic exposure to a set of environmental factors including pesticides, like rotenone, and herbicides. Familial PD implicates genetic susceptibility caused by one of several (point) mutations in the genes encoding for LRRK2, (leucine-

rich repeat kinase 2), DJ-1, PINK1, parkin, GBA (glucocerebrosidase gene, Gaucher's disease), UCH-L1, PODXL (podocalyxin-like), SYNJ1 (PARK20), ATP13A2, SNCA (α-synuclein) and others. Autosomal dominant mutations in the α-synuclein gene (SNCA) can lead to duplication or triplication of the gene, generating several copies of α-synuclein. A30P (G88C) and A53T are examples of PD linked point mutations in the α-synuclein gene involving the replacement of alanine by proline or threonine, respectively, at the indicated sites (Chen et al., 2015; Lee et al., 2010; Narhi et al., 1999; Ono et al., 2011; Park et al., 2015; Paumier et al., 2013; Stefanovic et al., 2015; Sudhaman et al., 2016; Vilageliu and Grinberg, 2017; Zhu et al., 2014).

Autosomal recessive mutations such as mutations in the GBA, parkin, PINK1, ATP13A2 and DJ-1 genes are likely linked to an early onset PD. Mutations in the parkin gene are the most common form of autosomal recessive PD. Parkin and Pink1 work together in regulating mitophagy, and in cases of mutations in parkin and PINK1, but also in ATP13A2, GBA and DJ-1, aberrant mitophagy is observed that leads to cell death. (Hanagasi et al., 2016; Lesage et al., 2016; Noelker et al., 2015; Park et al., 2015; Song et al., 2016; van der Merwe et al., 2015; Vilageliu and Grinberg, 2017).

The LRRK2 autosomal dominant mutation (G2019S) with gain of function is the most common known PD-associated gene and is also found in cases of idiopathic PD (Blanca Ramirez et al., 2017; Kalinderi et al., 2016). This mutation increases the risk to develop PD with 80% and it is linked to late onset PD. Curiously, the pathology in this case is independent of Lewy body formation (Gaig et al., 2009; Kalia et al., 2015).

Models to study PD

In order to investigate PD pathology, a variety of models have been created to address the clinical, tissue, cellular and molecular characteristics of PD.

Animal models and primary cell cultures are widely used. Primary cell culture is a fast way to study single neurons. Animal models may provide insight in the systemic toxicity of α-synuclein. Interestingly, investigations on α-synuclein trafficking showed that α-synuclein can be transported from the enteric system until the SN, where this protein accumulates and aggregates (Holmqvist et al., 2014). Obviously, this α-synuclein propagation could only be demonstrated in animal models with

experimentally induced PD since animals do not develop naturally neurodegenerative diseases like PD.

SH-SY5Y cell line (neuroblastoma) is one of the most frequently used cellular models to study PD. It is a human neuronal cell line that can be quickly and inexpensively differentiated into neuron-like cells. In addition, chronic exposure to neurotoxins or overexpression of different types of α-synuclein can mimic a PD phenotype. Nevertheless, the line is derived from a malignant tumor and, therefore, its basic physiology is altered. In the analyses of experiments with the SH-SY5Y cell line, there should be awareness that the SH-SY5Y derived neurons are incomparable to true, mature human mature neuron (Kovalevich and Langford, 2013).

S. cerevisiae (budding yeast) have been considered an important model to study the cellular biology, biochemistry and genetics of eukaryotes. This organism shows cellular pathways, proteins and genes that are well conserved during evolution (Smith and Snyder, 2006). Approximately 30% of yeast genes have known human ortholog genes, allowing studies on them with respect to the development of human diseases (Walberg, 2000). Furthermore, neurodegenerative diseases such as PD comprise the formation of protein aggregates, most often formed by protein misfolding processes. Mechanisms related to protein folding, oligomerization and aggregation can be studied in yeasts since protein quality control is conserved in these organisms (Ciaccioli et al., 2013; Khurana and Lindquist, 2010). Therefore, yeast humanized models to study PD are also very well accepted (Franssens et al., 2013).

Ten years ago, Yamanaka demonstrated that somatic cells could be reprogrammed into pluripotent stem cells (Takahashi and Yamanaka, 2006). The possibility to generate these induced pluripotent stem cells (hiPSC) from patients and to differentiate them into any cell type (so also the cell type that is specifically affected in that patient) has presented a unique tool for studying the development of neurodegenerative disorders apart from other applications (Figure 2). In order to address mechanisms underlying PD, researchers have started to differentiate hiPSC from PD patients into dopaminergic neurons for the analysis of pathogenesis of PD; it is obvious that PD iPSC-derived dopaminergic neurons provide a much more appropriate cell model than any of the PD cell models used so far. However, still a number of hurdles

have to be taken related to the genetic and epigenetic signatures still present in iPSC-derived cells. Moreover, much more efficient differentiation protocols for DA neurons and particularly for their purification still have to be developed (Devine et al., 2011; Jacobs, 2014; Kang et al., 2016; Marchetto et al., 2010).

It is clear that most experimental models for PD have their limitations and drawbacks. The researcher needs to choose the model that is the most appropriate and accurate to answer his specific detailed research questions.

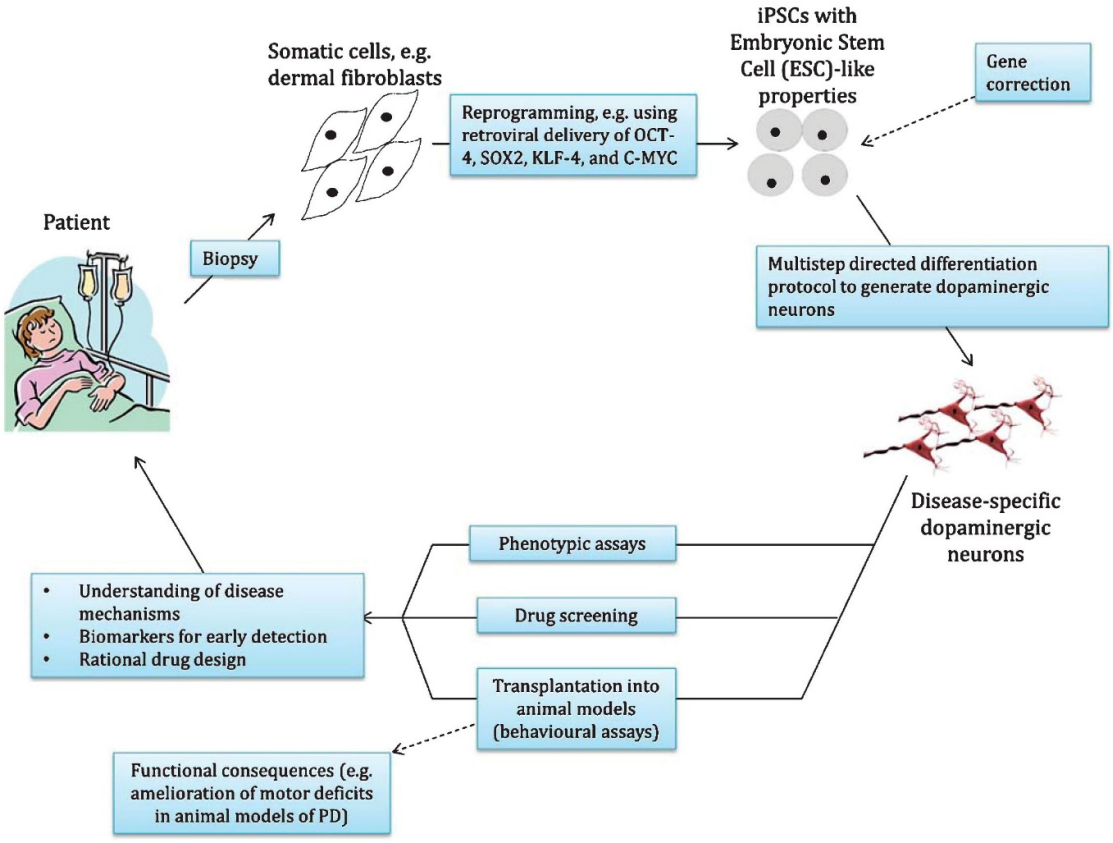

Figure 2: The iPSC paradigm for modeling PD and other neurodegenerative diseases (Jacobs et al., 2014).

OUTLINE OF THESIS

The degeneration of dopaminergic (DA) neurons associated with alpha-synuclein accumulation is a hallmark of Parkinson´s disease (PD). The overexpression

or the presence of mutated alpha-synuclein can lead to oxidative stress, aberrant autophagy and disturbed ER homeostasis. Several studies have addressed the initial cellular events occurring in the disease, suggesting that mitochondrial dysfunction, deficits in the intracellular axonal transport and alpha-synuclein aggregation are important events that could lead to cell death. Moreover, it has been suggested that proteins related to mitochondrial dynamics play a role in the mechanisms that lead to neurodegeneration in the presence of alpha-synuclein. However, the association between all these events and the role of proteins related to mitochondrial dynamics in PD are still unclear. After an extensive review of our present knowledge on the role of α-synuclein and mitochondrial dysfunction in the pathology of Parkinson's disease (**Chapter 2**), we report, in the chapters that follow, on studies addressing, registering and analyzing the disturbance of mitochondrial mobility and function in PD. In these studies, we made use of 4 different experimental cell models that are generally employed to mimic and study PD. In **Chapter 3**, we describe the effect of rotenone, a pesticide associated with sporadic PD, on mitochondrial mobility and motor protein expression in primary DA neurons. In the study in this chapter, we used cultured DA neurons isolated from the midbrain (the substantia nigra) of neonatal Lewis rats and exposed them to low doses of rotenone for 24h or 48h. Apart from analyzing mitochondrial mobility in these rotenone-treated DA neurons, we aimed to evaluate the effect of rotenone on the expression of several motor proteins involved in anterograde and retrograde mitochondrial trafficking. To investigate whether the changes in mitochondrial trafficking that we observed in the rotenone-affected primary DA neuron cultures also do occur in human DA neuron-like cells in which "familial PD" was triggered/mimicked by the expression of mutant alpha-synuclein genes (A53T or A30P), we employed a second, frequently used, PD cell model: the A53T- or A30P-gene transfected SH-SY5Y cell line which was induced to differentiate into neuronal-like cells (**Chapter 4**). In this chapter, we also investigated whether impaired mitochondrial trafficking and function can be rescued by NAP, a neuropeptide demonstrated to promote microtubule assembly. For that, we analyzed mitochondrial trafficking, distribution, connectivity and reactive oxygen species production in the mutant alpha-synuclein genes expressing differentiated SH-SY5Y cells with or without the NAP

treatment. In **Chapter 5**, we addressed the same questions regarding the PD-related pathogenesis of mitochondrial mobility and function as in the previous chapters, but with the use of a third PD cell model: iPS cells generated from skin fibroblasts of patients with familial forms of PD, i.e. patients with a triplication of the alpha-synuclein gene (SNCA3) or patients with a mutation (A53T) in the alpha-synuclein gene. These induced pluripotent stem (iPS) cells were differentiated into DA neurons after which the effect of aberrant alpha-synuclein expression on mitochondrial trafficking, morphology and distribution was analyzed.

The maintenance of mitochondrial dynamics is dependent of Miro. The best way to investigate whether Miro could play a role in the impairment of mitochondrial dynamics caused by alpha-synuclein is deleting the Miro gene. However, since neurons are polarized cells that depend on normal mitochondrial trafficking to survive, yeasts appeared to be an excellent model to study the role of Miro, since mitochondrial dynamics in yeast cells is not dependent of intracellular trafficking. So, the fourth experimental cell model used by us (**Chapter 6**), was the humanized yeast model with the knockout of Miro (Δ*Gem*) and the forced expression of point-mutated A30P and A53T alpha-synuclein. In the yeast model, we assessed the role of Miro in the (aggregated) alpha-synuclein induced changes in cellular viability, mitochondrial and autophagy dysfunction and endoplasmic reticulum (ER) stress. Finally, in **Chapter 7**, we summarized and discussed the findings presented in the preceding chapters.

Our findings indicate that aberrant alpha-synuclein expression disrupts axonal trafficking of mitochondria leading to mitochondrial dysfunction, disturbed ER dynamics and aberrant autophagy through mechanisms dependent on Miro.

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CHAPTER 2

ALPHA-SYNUCLEIN EFFECTS UPON INTRACELLULAR TRAFFICKING AND CELLULAR STRESS IN PARKINSON´S DISEASE

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Abstract

Parkinson's disease (PD) is the most common motor neurodegenerative disease in the world. Protein aggregates containing mainly alpha-synuclein and mitochondrial dysfunction are a hallmark of disease. Alpha-synuclein leads to a global cellular toxicity and affects degradation systems, unfold protein response and energy production, impairing the cellular redox balance. Mitochondria and autophagy defects have been suggested to be caused or caused by alpha-synuclein accumulation. In this study we reviewed the role of alpha-synuclein and proteins related to mitochondria, endoplasmic reticulum and autophagy dynamics in the pathogenesis of PD.

INTRODUCTION

Parkinson's disease (PD) was initially described by James Parkinson in 1817, and is currently the most common age-related neurodegenerative movement disorder (Goetz, 2011; Parkinson, 2002; Tanner, 1992). There are at the present time approximately 10 million diagnoses worldwide, and PD affects 2% of the population aged >60 years. Additionally, the Parkinson's Disease Foundation (2016) reports that men are 1.5 times more likely to have PD than women do. Clinical symptoms of PD include motor dysfunctions such as muscle rigidity, bradykinesia, balance disturbances, and resting tremor, and non-motor symptoms such as cognitive decline, depression, and olfactory and gustatory deficits in the early stages of the disease. Mood alterations, sleep disturbances, and dementia also typically occur in late PD (Cecchini et al., 2015; Weintraub et al., 2008).

Studies of post-mortem brains from patients with PD have revealed the presence of cellular inclusions called Lewy bodies, which contain α-synuclein. Accumulation of α-synuclein in both neurons and glia precedes degeneration of dopaminergic (DA) neurons located in the substantia nigra (SN). Therefore, α-synuclein aggregation is considered a hallmark of both sporadic and familial PD and the protein has been extensively investigated. α-Synuclein damaging cells seems to involve a global toxicity that is time-dependent and impairs mitochondria, lysosome and other organelles and compartments proper functioning. However, molecular mechanisms of α-synuclein action in these organelles and compartments are not well understood.

The current review presents an overview of the mechanisms of PD pathogenesis related to α-synuclein toxicity, focusing on intracellular impairment and oxidative stress.

Characteristics of α-synuclein

α-Synuclein is a small protein composed of 140 amino acids; it is a neuronal presynaptic protein critically involved in recycling vesicles at synapses, including their trafficking, docking, and endocytosis at the presynaptic membrane. It is also expressed at low levels in the soma, dendrites, and axons and is essential to the regulation of neurotransmitter release and responses to cellular stress (Bengoa-Vergniory et al.,

2017). In DA neurons specifically, α-synuclein is important for the synthesis, regulation, storage, and release of dopamine; it is a crucial protein for synaptic plasticity (Burre, 2015). Moreover, α-synuclein interacts with numerous membrane lipids and proteins, such as those related to calcium and dopamine homeostasis (Ruzafa et al., 2017). In addition, excess of α-synuclein interacts with tyrosine hydroxylase (TH) to inhibit dopamine biosynthesis, and with the dopamine transporter (DAT) impairing dopamine transport (Khan et al., 2012). Overexpression of wild type (WT) α-synuclein or expression of the mutated A53T form reduces dopamine release via covalent binding to dopamine and/or modulation of TH; this disrupted dopamine metabolism leads to cell toxicity (Tabrizi et al., 2000; Xu et al., 2002).

Oligomerization and aggregation of α-synuclein

Proteins normally fold to form three-dimensional structures in cells. However, incorrect folding may occur, causing misfolding or incomplete folding. These aberrant proteins may be prone to aggregate and spread (Mahul-Mellier et al., 2015).

Overexpression or anomalous conformation due to point mutations and a high oxidative environment lead to oligomerization of α-synuclein and formation of amyloidogenic filaments. These events subsequently lead to formation of aggregates and Lewy bodies (Li et al., 2004; Piccirilli et al., 2017). Furthermore, α-synuclein may form multimers by self-assemblage, which irreversibly produces insoluble aggregates. Mutant A53T α-synuclein appears to be more prone to aggregate (including dopamine) than A30P or WT α-synuclein are (Cieplak, 2017; Moussa et al., 2008). The role of protein aggregation is unclear; however, neurotoxic effects of α-synuclein have been demonstrated to result from its propensity to form multiple oligomers. In contrast, other studies indicate that oligomers of α-synuclein (pre-aggregates) are more toxic than aggregates (Deas et al., 2016; Lam et al., 2016; Lorenzen and Otzen, 2014; Winner et al., 2011), and aggregate formation has also been suggested as a neuroprotective mechanism (Arawaka et al., 2014; Mahul-Mellier et al., 2015).

Insoluble α-synuclein may result from genetic mutations, deficits in degradation, and exposure to oxidative conditions (Conway et al., 1998; Follmer et al., 2015; Fredenburg et al., 2007; Hashimoto et al., 1999; Narhi et al., 1999). However, α-

synuclein elimination may prevent insoluble protein formation (Myohanen et al., 2017).

The monomeric form of the protein is predominantly degraded by chaperone-mediated autophagy (CMA); nevertheless, in few cases or in the presence of ser-129 phosphorylated α-synuclein can be degraded by proteasome. The oligomeric form of the protein is degraded by autophagy, independent of CMA (Machiya et al., 2010; Vogiatzi et al., 2008). However, mechanisms that favor oligomeric and insoluble α-synuclein formation as a component of PD-associated neurodegeneration are not well understood. The general mechanisms of α-synuclein degradation have been studied and the findings indicate that small inclusions of the protein and puncta aggregates are ubiquitinated and driven to the ubiquitin-proteasome system. However, proteasomes do not degrade large cargos such as aggregates or organelles; degradation of ubiquitinated or non-ubiquitinated cargos, including aggregated α-synuclein, occurs preferentially through autophagy (Ciechanover et al., 2000; Lynch-Day et al., 2012).

Macroautophagy is the most well characterized autophagy process. During macroautophagy, macromolecules and organelles are isolated in the cytosol by a double membrane that forms a vesicle, termed autophagosome, which matures and fuses with lysosome (Klionsky, 2005). P62 protein recognizes ubiquitinated cargos and initiates autophagy by binding to microtubule-associated protein light chain 3 I (LC3-I) (an Atg8 yeast ortholog), which drives vesicle formation. When the autophagosome matures, LC3-I is converted into LC3-II. During the fusion process, LC3-II from the outer membrane fuses with the lysosome and is degraded with P62 and the vesicle. Several studies have assessed autophagic flux and cargo removal by measuring LC3-I, LC3-II and P62 levels (Erustes et al., 2017; Mizushima et al., 2010).

Both mutated and overexpressed wild-type α-synuclein disrupts the ubiquitin-proteasome system and autophagy; interestingly, dysfunction of degradation pathways also leads to accumulation and aggregation of α-synuclein. During aging and in the presence of large amounts of α-synuclein or mutated A53T α-synuclein, CMA and macroautophagy are inhibited. Furthermore, autophagy inducers decrease α-synuclein expression, indicating that autophagy is crucial for α-synuclein degradation and for preventing aggregate formation (Dagda et al., 2013; Decressac et al., 2013; Shruthi et al., 2016; Song et al., 2014; Wu et al., 2013).

Mitochondrial dysfunction may result from aggregation and lysosome dysfunction. In the presence of mutated A53T α-synuclein, autophagy is blocked and endoplasmic reticulum (ER) homeostasis is disturbed, thereby increasing reactive oxygen species (ROS) levels also caused by mitochondrial dysfunction. Since autophagy has a global role of degradation in the cell, inhibition of this process by A53T α-synuclein, can lead to high ROS levels via ER impairment and reduced mitochondria removal. Especially in DA neurons, defects in non-functional mitochondria degradation causes a rapid increase in ROS levels and induce neurodegeneration (Hattori et al., 2017; Redmann et al., 2016). Together, these studies show that α-synuclein toxicity impairs degradation pathways, thereby worsening the consequences of protein accumulation and aggregation as depicted in Figure 1.

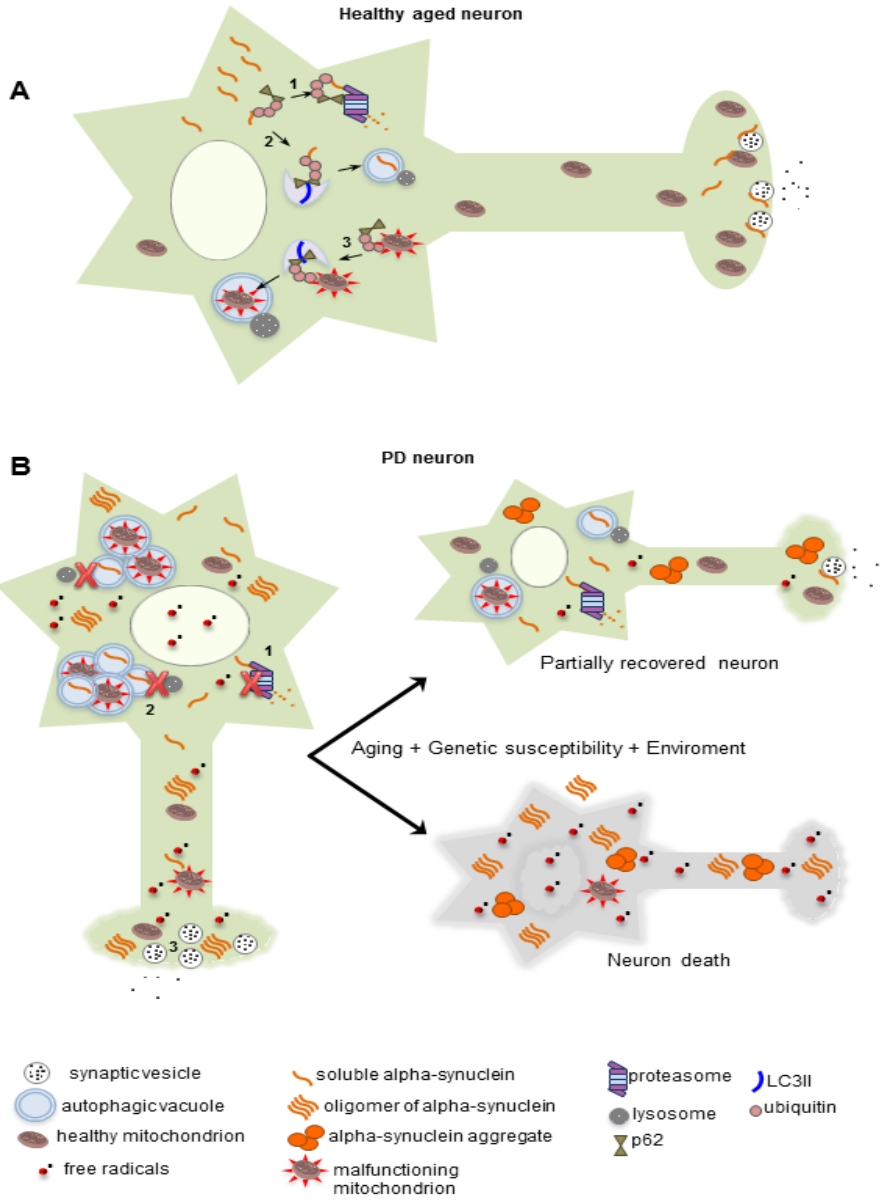

Figure1: Soluble monomeric α-synuclein and oligomers and aggregates in normal aged neuron (A) and neurons with PD (B). α-Synuclein oligomers toxicity involve the impairment of degradation pathways via proteasome or lysosome worsening the consequences of protein accumulation and aggregation. α-Synuclein aggregates alleviate the cellular stress caused by oligomers, however in long-term the formation of more oligomers and aging, genetic susceptibility and environment factors lead to DA neurons death.

Toxicity of α-synuclein in dopaminergic neurons

Experiments with both A53T and A30P and other mutated forms of α-synuclein reveal that the oligomeric ß-sheet-rich secondary structure of α-synuclein is highly toxic

and is also crucial to PD pathology (Dettmer et al., 2015a, b; Ranjan and Kumar, 2017; Sharon et al., 2003). During PD pathogenesis, soluble oligomers with high membrane-binding affinities may spread among neurons and glia, with the seeding oligomers acting like prions (Abeliovich and Gitler, 2016; Ugalde et al., 2016). According to this theory, PD is considered a prion-like disease, comparable to diseases such as bovine transmissible spongiform encephalopathy in animals and Creutzfeld-Jacob disease in humans. Studies on post-mortem brains of patients with PD who received fetal tissue grafts 10–15 years ago, have found Lewy body-like structures in the graft tissue, suggesting that α-synuclein entered the graft cells from the surrounding host cells, similarly to the mechanisms of prion spreading (Danzer et al., 2009; Li et al., 2008; Rey et al., 2016).

Experiments using animal models to study the prion-like properties of α-synuclein indicate that animals treated with α-synuclein fibrils developed α-synuclein aggregates and synucleinopathy, including concomitant deficits in synaptic function. Holmqvist and collaborators (2014) revealed that monomeric, oligomeric, or fibrillar α-synuclein is transported from the enteric system to the brain via the vagus nerve, via the slow and fast intracellular microtubule transport systems. These findings suggest an intimate relationship between α-synuclein translocation and aggregate formation.

The role of reactive oxygen species in Parkinson's disease

During PD, the activities of mitochondrial complexes I and IV are preferentially affected in the SN, thereby increasing ROS production. ROS can be generated by various cellular processes such as α-synuclein accumulation or the presence of mutated proteins in cellular compartments such as the endoplasmic reticulum (ER) during cellular stress. Notwithstanding, ROS are produced primarily via the electron transport chain, at the inner mitochondrial membrane (Blesa et al., 2015; Penke et al., 2016; Santos et al., 2009; Turrens, 2003).

Mitochondria are crucial for the maintenance of healthy neurons, since these organelles are responsible for producing energy, in the form of ATP. Moreover, mtDNA encodes 7 essential proteins involved in the respiratory chain (Devi et al., 2008; Mootha et al., 2003). Mitochondria are also involved in regulating apoptosis and in calcium and

ROS homeostasis. In healthy mitochondria, the electron chain generates a molecular cascade respiratory chain. This cascade induces a gradient of protons over the mitochondrial inner membrane, which is used in ATP synthesis. Electrons are extracted from reduced substrates and are transferred to molecular oxygen (O_2), through a chain of enzymatic complexes (I to IV). In the last step of the electron transport chain, cytochrome c oxidase (complex IV) completely reduces O_2 in water, with minimum formation of oxygen radicals. However, during mitochondrial dysfunction, partial reduction of O_2 occurs more frequently than normal reduction, thereby generating increased radical superoxide anions; approximately 0.1–0.5% of O_2 are partially reduced by mitochondria. The radical superoxide anion can be dismuted in H_2O_2 and O_2 by the Cu/Zn-SOD1 enzyme in the intermembrane space, or in the mitochondrial matrix by $MnSOD_2$ (Arun et al., 2016; Lopert and Patel, 2016; Quiros et al., 2016).

Mitochondrial dysfunction occurs during normal aging, leading to higher ROS production that accelerates α-synuclein aggregation and dopamine depletion, thereby initiating neurodegeneration (Benigni et al., 2016; Kong et al., 2014; Navarro and Boveris, 2010). DA neurons in the SN compacta (SNc) are susceptible to aging-associated oxidative stress. This susceptibility to ROS has been explained using the free radical theory, based on a hypothesis formulated in 1950 by Denham Harman (Harman, 2009). According to this theory, accumulated ROS damages macromolecules, thereby resulting in neurodegeneration. During aging, the redox state of the brain is disturbed by reduced antioxidants such as GSH (glutathione), which produces ROS toxicity and the resultant genetic mitochondrial mutations, protein damage, and ultimately, the development of neurodegenerative diseases such as PD (Currais and Maher, 2013; Kudo et al., 1990; Zuo and Motherwell, 2013).

Mitochondrial dysfunction in Parkinson's disease

Human DA neurons are considered the greatest consumer of ATP since they form 2.4 million synapses and they are thought to be the neurons with more synapses connections (Haddad and Nakamura, 2015). Thus, mitochondria dysfunction inevitably contributes to ROS increases in DA neurons, especially given that 0.2–2% of total oxygen consumed in normal conditions is converted to free radicals in the mitochondria

(Maharjan et al., 2016; Richter, 1992).

During the process of energy production by mitochondria, unpaired electrons are formed, mainly in the complexes I and III. The presence of these unpaired electrons facilitates ROS production, which can accelerate aging and activate antioxidant enzymes, including SOD and GPx, and antioxidant components including DAT and vesicular monoamine transporter 2 (VMTA2), which drives dopamine relocation from the intracellular medium to synaptic vesicles (Forkink et al., 2015; Oka et al., 2015). Several studies propose that complex I disruption increases ROS levels in the mitochondrial matrix and the cytosol, leading to GSH depletion and cell death. In addition, ROS levels rapidly increase throughout tissues, including the PD brain and platelets of PD patients in cybrid models (Arduino et al., 2015; Bronstein et al., 2015).

The mitochondrial electron transport chain produces 90% of ROS in cells and is localized close to mtDNA, thereby facilitating mtDNA mutations. Furthermore, mtDNA is not protected by histones and its replication is independent from the cell cycle. Consequently, mutated mtDNA needs to be repaired quickly to avoid the propagation of these mutations that can lead to somatic mosaicism, a risk increasing with age. Recently, mitochondrial DNA damage was linked to complex I deficiency and increased ROS in PD (Fayet et al., 2002; Giannoccaro et al., 2017; Leman et al., 2015).

Compared to other tissues, the brain is more susceptible to mtDNA mutations, especially in the SN. A study of healthy 60-year-old participants found that >40% of all mtDNA deletions occur in the SN (Ameur et al., 2011; Bender et al., 2006). Mutations in mtDNA and/or electron transport chain impairments lead to mitochondrial dysfunction and energy depletion. Decreased energy production compromises the repair of damaged mitochondria and mitochondrial quality control, making mitochondria an easy target for degradation (mitophagy) (Lauri et al., 2014). Damaged or depolarized mitochondria cause electron leakage, generating excessive ROS, and releasing pro-apoptotic factors such as cytochrome C, which initiate cell death (Brustovetsky et al., 2002). Moreover, electron chain inhibitors impair complex I and can interact with α-synuclein, especially with mutated forms of the protein, increasing ROS production. Curiously, basal expression of α-synuclein has been shown to protect against excessive ROS generation (Byers et al., 2011; Choong and Say, 2011).

Mitochondria form a highly interconnected network throughout the neuron and its dynamics involves continuous autophagic destruction via a macroautophagy process, termed mitophagy. Maintenance of healthy mitochondrial functioning involves fusion and fission processes that alter mitochondrial morphology (Bereiter-Hahn and Voth, 1994; van der Bliek et al., 2013). Deficits in the fusion or fission machinery cause aggregation and loss of directed movement, thereby impairing correct mitochondrial migration to neurites. Furthermore, investigations of impaired fusion and fission have demonstrated spontaneous generation of mtDNA mutations in neurodegenerative disorders such as PD (Chen and Chan, 2009).

Mitochondrial membrane damage and changes in mitochondria fission and morphology can result from interactions with α-synuclein (Nakamura et al., 2011). In order to understand α-synuclein toxicity and analyze the consequences for mitochondrial dynamics, several models have been created to overexpress α-synuclein or transfect mutated forms of the protein. It was revealed that overexpression of α-synuclein inhibits mitochondrial membrane fusion and disturbs the mitochondria cycle, which leads to fragmented or swollen mitochondria that contain laminated bodies. Moreover, α-synuclein overexpression increases colocalization of autophagosomes and mitochondria. Interestingly, siRNA-mediated α-synuclein knockdown prevents changes in mitochondrial morphology that results in elongated mitochondria (Kamp et al., 2010; Martin et al., 2006; Ryan et al., 2015).

Mutations in α-synuclein may also amplify mitochondrial dysfunction. A53T α-synuclein colocalizes to the mitochondrial membrane disrupting complex I and interfering with the fission process and autophagy machinery (Pozo Devoto and Falzone, 2017). However, mitophagy is blocked, leading to the appearance of fragmented mitochondria. In addition, damaged mtDNA and dysmorphic mitochondria occur in A53T α-synuclein transgenic mice (Chinta et al., 2010; Choubey et al., 2011; Martin et al., 2006), and may have resulted from altered affinity of mutated α-synuclein to the mitochondrial membrane, given that α-synuclein primarily interacts with the outer mitochondrial membrane. However, during adequate ATP supply and pH changes, α-synuclein can migrate to the inner mitochondrial membrane quickly changing mitochondrial membrane potential, inhibiting complex I, and leading to aggregation and

fragmentation of mitochondria (Cole et al., 2008; Devi et al., 2008).

Interaction between mitochondria and ER in α-synuclein toxicity

A proper mitochondrial membrane potential is important for maintaining a normal ER morphology. Altered mitochondrial membrane potential is associated with ER fragments that release calcium and produce high intracellular calcium levels, which leads to increased ROS. These findings suggest that both ER stress and mitochondrial dysfunction contribute to DA degeneration. Interestingly, mitochondria morphologic changes, caused by interaction of α-synuclein with the mitochondrial membrane, are exacerbated by A53T α-synuclein. In contrast, A30P α-synuclein does not exacerbate these changes, since this protein does not interact with the mitochondrial membrane (Bao et al., 2016; Ghio et al., 2016).

The findings discussed above suggest that mitochondria and ER are affected by α-synuclein toxicity; however, further research is needed to understand crosstalk between these organelles in order to further elucidate their dysfunction in PD. Mitochondria and ER possess mutual membrane contact sites, which allow direct contact for metabolite exchange, for signaling involved in organelle dynamics, ATP metabolism, protein folding, and autophagy (van Vliet et al., 2014; Vance, 2014; Vishnu et al., 2014). Moreover, both organelles form contacts at synapses, where they promote calcium flow and synaptic activity (Krols et al., 2016; Mironov and Symonchuk, 2006). Together, these findings strongly suggest that contacts between mitochondria and ER are essential to neuron survival. Furthermore, mitochondrial fission processes occur near contact sites with the ER even in the absence of mitochondrial fission factors. Besides that, the mitofusins, MFN1 and MFN2, which are proteins involved in mitochondrial fusion, depend on Miro1, a crucial protein associated with mitochondrial trafficking and dynamics. Miro1 is localized at sites of contact between ER and mitochondria, and the yeast Miro1 ortholog, Gem, participates in mitochondrial and ER division through interactions with the membrane contact sites between organelles (Fransson et al., 2006; Misko et al., 2010; Rowland and Voeltz, 2012). Furthermore, mitophagy also appears to be dependent on mitochondrial and ER membrane contact sites. Several ATG proteins, including the Atg8 mammalian ortholog LC3, are found at mitochondrial and ER contact sites. Moreover, in yeast or mammalian cells,

mitochondrial and ER contact sites form a platform for mitophagosome biogenesis and mitochondrial degradation (Bockler and Westermann, 2014; Hamasaki et al., 2013).

Investigations of mitochondria and ER contacts in PD indicate that α-synuclein is located at mitochondria and ER contact sites. Furthermore, α-synuclein overexpression increases mitochondria and ER contacts and affects calcium transfer between these organelles. However, in the presence of the A30P or A53T mutated α-synuclein oligomers, contacts between both organelles are inhibited even more when Lewy bodies are present (Cali et al., 2011; Guardia-Laguarta et al., 2014). This diminished organelle contact induces defective mitochondrial fission, and overexpressed or mutated α-synuclein leads to blockage of autophagy, and mitochondrial accumulations can be found (Manor et al., 2015). Therefore, inhibited autophagy also increases ROS levels via damaged mitochondria and ER stress, which eventually lead to cell death.

There is a reciprocal relationship between mitochondrial dysfunction and ER stress. Post-mortem brains from patients with PD and animal models of PD both showed indications of ER stress. Overexpressed or mutated α-synuclein accumulates in the ER impairing protein folding and provokes ER stress. Furthermore, A53T α-synuclein increases ROS levels by impairing mitochondria and ER function, thereby causing neuron death (Colla et al., 2012; Smith et al., 2005). Stressed ER also generates ROS by decreasing GSH levels and transferring excessive calcium to mitochondria, which then also generate more ROS. GSH is the main molecule responsible for maintaining redox states in ER and mitochondria. Moreover, GSH oxidizes and activates the unfold protein response (UPR). In order to restore ER homeostasis, inositol-requiring enzyme 1 alpha protein (Ire1α) activates the UPR, which subsequently requires chaperones such as protein disulfide isomerase (Pdi); this increases the folding and secretion of proteins to be degraded hence reestablishing the ER redox state. Secretion of α-synuclein also promotes its own accumulation and contributes to Lewy body formation. Furthermore, α-synuclein, disrupt both the ubiquitin-proteasome system and autophagy, leading to ER stress and UPR activation (Brigelius-Flohe and Maiorino, 2013; Schroder, 2008).

Pdi is the primary protein involved in the ER protein folding machinery, and is

overexpressed during ER stress; therefore, it is a marker for ER stress. During the protein folding process, Pdi oxidizes the protein generating disulfide bonds in proteins that become reduced. ER oxidoreductase 1 (Ero1) enzymatically oxidizes Pdi, which reactivates it for another cycle of protein folding. Once reduced, the protein Ero1 transfers oxygen to molecular oxygen generating H_2O_2. In addition, accumulated protein in the ER favors calcium leakage to cytosol. Mitochondria also play a role in calcium buffering; uptake of excessive calcium released from the ER increases mitochondrial metabolism and ROS production. Furthermore, protein folding, which occurs in the ER, requires high levels of ATP. Therefore, prolonged UPR activation promotes high levels of ROS as illustrated in Figure 2 (Feissner et al., 2009; Malhotra and Kaufman, 2007).

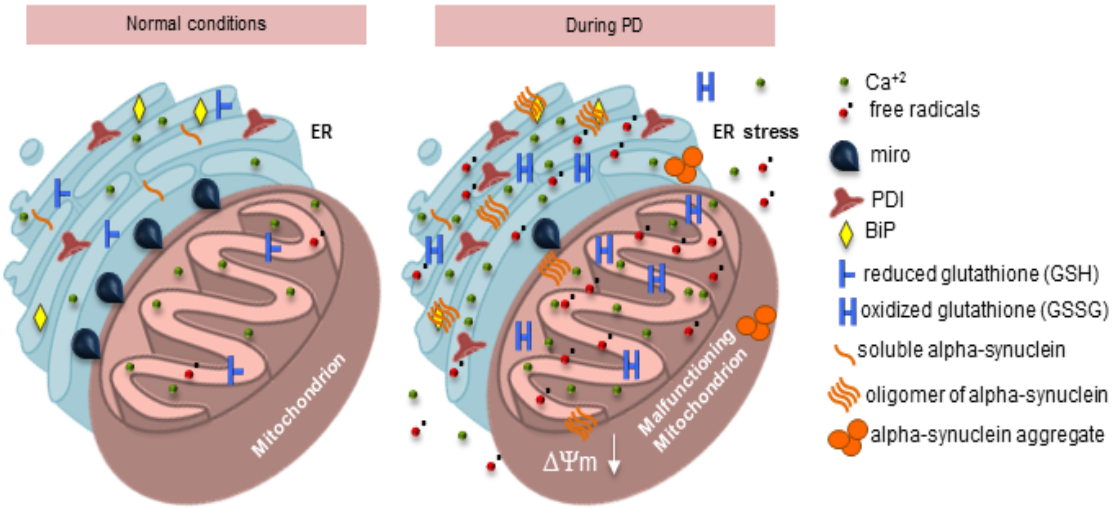

Figure 2: Effects of prolonged UPR activation by α-synuclein. Illustration of how α-synuclein affects the number of contact sites mediated by Miro, between ER and mitochondria in normal healthy or unhealthy neurons. During PD, the levels of chaperone PDI increased while, the levels of chaperone BiP (HSP70) decreased, both changes contributes to increase the consumption of GSH, leading to increased levels of GSSG, calcium and free radicals leading to DA neurons death.

In DA neurons, UPR activates *XBP1*, yeast ortholog *Hac1,* which plays a role in activating gene expression to promote neuron survival. However, in the long-term presence of excessive or misfolded proteins, Hac1 (mRNA) activates apoptotic genes such as transcriptional factor CHOP (*DDIT3* gene) that drives neurons to death. Besides that, Ire1α promotes alternative splicing of Hac1 mRNA, which is also a marker of ER stress. Therefore, UPR plays a paradoxical role in neurons; it initially activates mechanisms to ameliorate ER stress, however, long-term UPR activation induces cell death (Delic et al., 2012; Grimm, 2012; Krols et al., 2016; Mercado et al., 2016; Nikawa et al., 1996).

Recent studies of the first steps in neurodegeneration indicate that alterations in intracellular trafficking are essential to neuron survival. Experiments in cells with varying α-synuclein expression, without aggregates, revealed that high levels of this protein are associated with hyperphosphorylation of tau protein, which results in destabilization of microtubules and impaired intracellular trafficking of vesicles and

organelles (Oikawa et al., 2016). These findings suggest that alterations in intracellular trafficking are in fact initial steps in neurodegeneration and may promote aggregate formation.

Impaired intracellular trafficking in PD

Mitochondrial quality control is essential to neuronal survival and involves trafficking mitochondria to neuronal regions that require more energy and returning mitochondria to the soma for recycling and repair, since that is where fusion and fission preferentially occur. The axons of DA neurons in the SN account for 95% of the cellular volume and recruit a significant portion of its energy. Disrupted mitochondrial trafficking impairs the ATP-supply at specific sites, such as synaptic terminals, and impairs new healthy mitochondria generation by fusion and fission processes in the soma (Phillipson, 2017). Anterograde mitochondrial trafficking is the axonal transportation of mitochondria from the soma to the synaptic terminals. Retrograde transport to the soma is required during recycling, or in cases of mitochondrial damage and dysfunction. At the soma, mitophagy involves lysosomes and ubiquitin-proteasome processes to degrade damaged mitochondria (Florenzano, 2012; Lehmann et al., 2016). Since neurons are polarized cells and possess long axons, intracellular trafficking is crucial to neuronal survival, morphology, and function. Motor proteins from the kinesin family (KIFs), and other proteins such as dynein and dynactin, are responsible for maintenance of intracellular trafficking along the microtubules (Hirokawa et al., 2010). The direction of trafficking depends on the polarity of specific sites. Microtubule polarity is positive in the axon endings and distal dendrites; however, polarity may be either positive or negative in the proximal dendrites. In the soma, microtubular polarity is positive at the distal portion, after the microtubule-organizing center (MTOC) (Xiao et al., 2016).

Injuries to the cytoskeleton are responsible for rearrangement and movement of organelles in neurodegenerative diseases, including PD. Microtubules participate in diverse cellular functions including motility, cell division, and transportation of organelles, vesicles, and proteins, and maintenance of cellular morphology and general organization of the cytoplasm. Microtubular dynamics are regulated by the

concentration of free tubulin. Intriguingly, in PD, α-synuclein and Lewy bodies are colocalized with free tubulin and with the tubulin polymerization promoting protein (TPPP), suggesting that α-synuclein may disrupt intracellular trafficking by impairing microtubule stabilization (Morris and Hollenbeck, 1993; Olah et al., 2011; Szunyogh et al., 2015).

Accumulating evidence suggests that disrupted axonal transport is critical to PD development (Hunn et al., 2015). It has been suggested that α-synuclein may impair mitochondrial axonal transport by disturbing motor protein expression such as for dynein. In addition, α-synuclein appears to disrupt interactions of these proteins with microtubules (Cartelli et al., 2016; Fang et al., 2017). Other studies have demonstrated that during neurodegeneration, alterations in motor proteins may have consequences for mitochondrial trafficking (Cai et al., 2005). Expression of the anterograde motor proteins KIF1Bα and KIF5 and the retrograde motor proteins dynein, dynactin, and syntaphilin, was altered prior to protein aggregation in cell cultures and in animals treated with rotenone, a pesticide which blocks complex I of mitochondria electron chain. In addition, mitochondrial trafficking is synchronized with anterograde motor protein expression, and alterations in these proteins change mitochondrial trafficking (Chaves et al., 2013; Melo et al., 2013). Together, these studies strongly suggest that altered intracellular trafficking is an important component of PD pathogenesis.

To transport cargos such as mitochondria, motor proteins associate with the adaptor proteins Trak (drosophila ortholog Milton) and Miro (also called Rhot), which are attached to the outer mitochondrial membrane (Devine et al., 2016). Experiments altering Miro expression demonstrate that increased Miro increases mitochondrial trafficking, indicating regulation of mitochondrial dynamics (Chen and Sheng, 2013). In addition, loss of Miro results in defective trafficking in both directions, suggesting that Miro is an adaptor for both anterograde transport (via interaction with KIF5) and retrograde transport (via interaction with dynein). Furthermore, studies on mitochondrial fragmentation and interconnectivity showed that non-functional Miro led to mitochondrial trafficking impairments and fragmentation, whereas overexpression of Miro increased mitochondrial trafficking and interconnectivity, thereby increasing mitochondria length in neurons (Fransson et al., 2006; MacAskill and Kittler, 2010).

Miro is a Ca^{+2} sensor containing 2 calcium-binding EF-hands. Increased calcium dissociates motor proteins from Miro and Trak, blocking mitochondria trafficking. This process is crucial for the anchoring mitochondria at specific sites where abundant ATP is required, such as at synapses. When ADP decreases, stationary mitochondria move to another site with low ATP levels. However, impaired mitochondrial trafficking can lead to mitochondrial dysfunction at the current anchor site and result in increased ROS generation (see Figure 3) (Klosowiak et al., 2013; Mironov, 2007; Saotome et al., 2008).

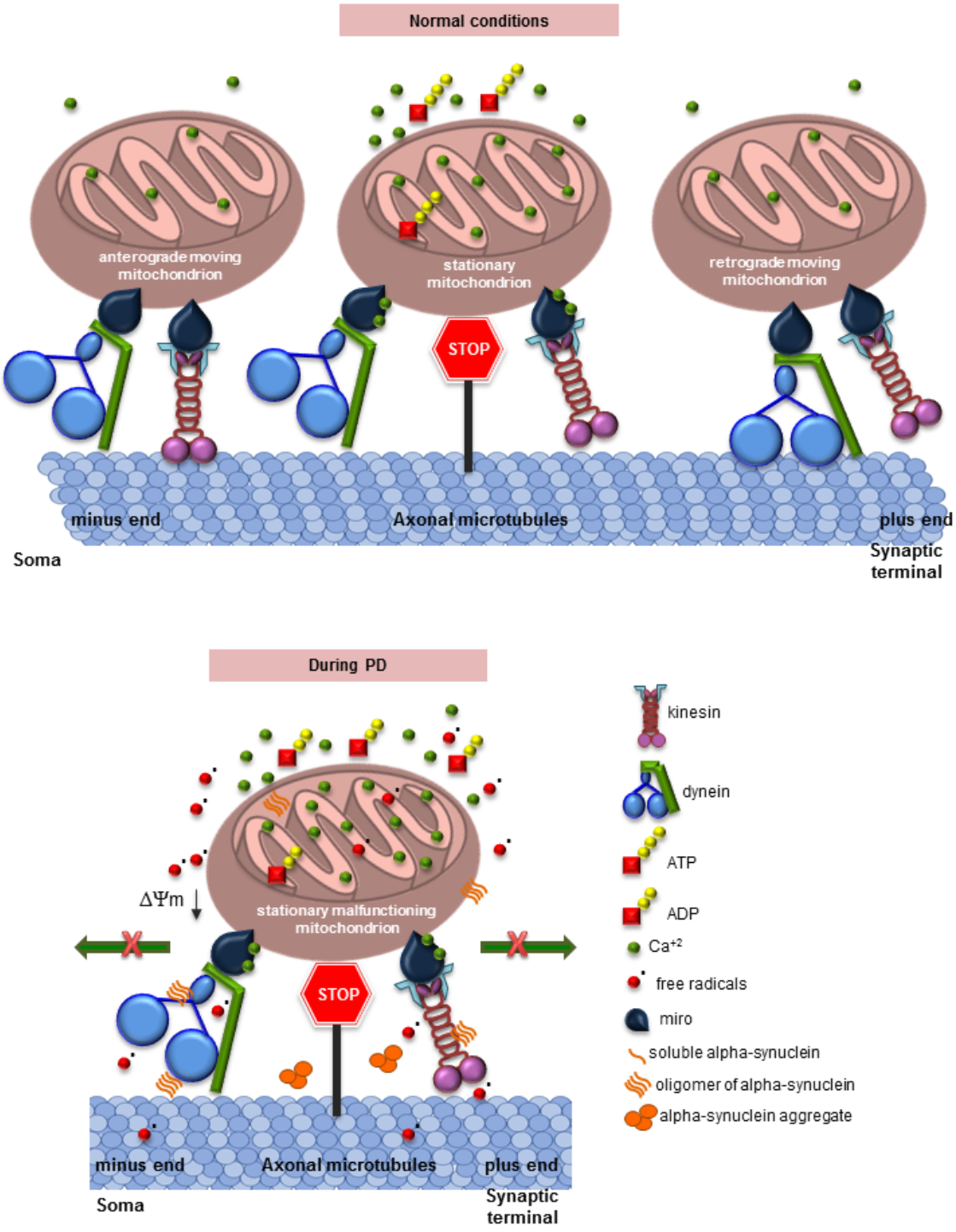

Figure 3: Illustration of mitochondria trafficking and anchoring in sites where ATP is required in healthy neurons (normal conditions) and during PD (PD). In the presence of α-synuclein the mitochondrial anterograde and retrograde trafficking are impaired and mitochondrial dysfunction is worsen increasing the levels of free radicals and calcium contributing to decreased axonal trafficking.

Miro also interacts with mitofusin (Mfn) proteins, which participate in mitochondrial fusion. Intriguingly, mitochondrial trafficking is decreased in neurons in

which *MFN2* was knocked-out, suggesting that Miro and Mfn work together in the regulation of mitochondria trafficking (Saotome et al., 2008). Miro is associated with the mitochondrial outer membrane; it coordinates the transport of mitochondria moving together with ER and it takes care that mitochondria stay close enough for the initiation of fusion or fission processes (Friedman et al., 2010). Once in contact, Mfn1 and Mfn2 interact with Miro and both are required for proper axonal transport, suggesting that association of these proteins and balanced trafficking are essential for the fusion process (Misko et al., 2010). Absence of Miro exacerbates mitophagy, and transgenic *MFN2* knockdown mice demonstrated blocked mitophagy. The ER provides lipids to form membrane vesicles during autophagy (Axe et al., 2008) these lipids are transferred and accumulate at the outer mitochondrial membrane before they are transported to the sites of vesicle formation and mitophagy initiation (Hailey et al., 2010). However, alterations in Miro or Mfn proteins can disturb these processes, revealing that both proteins are required for normal fusion and mitophagy processes.

Damaged mitochondria are targeted for mitophagy via PINK1 signaling. Parkin forms a complex with PINK1 during mitophagy and ubiquitinates substrates at the outer mitochondrial membrane, including Miro, which triggers mitophagy. Miro may function as a receptor for both proteins, since Miro interacts with PINK1 and parkin, thereby allowing their association with the outer mitochondrial membrane. Moreover, damaged mitochondria require rapid Miro ubiquitination, which is mediated by parkin. In addition, the fibroblasts of patients carrying parkin mutations showed altered Miro turnover, suggesting that Miro is critically involved in regulating fusion, fission, and mitophagy events (Birsa et al., 2014; Kazlauskaite et al., 2014).

As discussed previously, α-synuclein toxicity in the ER and mitochondria can change calcium levels in cytosol. However, the mechanisms underlying this process in PD have not been elucidated. Furthermore, Dučić (2015) demonstrated that α-synuclein plays a role in the regulation of intraneuronal calcium levels. Together, these findings suggest that α-synuclein impairs mitochondrial dynamics and mitophagy, leading to disrupted mitochondrial trafficking via Miro signaling.

The specificity of intracellular organelle trafficking among cellular compartments is strictly regulated by small GTPases (Rabs) from the Ras super family

of proteins. Furthermore, Rabs are responsible for the correct attachment of motor proteins and cargos and for cargo motility and their delivery to the correct destination. Furthermore, Rabs may be involved in α-synuclein toxicity, such as through formation of Lewy bodies, as discussed above. Cultured cells internalize α-synuclein added to the culture media, which is associated with aggregate formation. α-Synuclein is secreted from cells via exocytosis, and subsequently internalized by other neurons in culture via Rab5-dependent endocytosis, thus initiating a spreading cycle of seeding α-synuclein and aggregate formation (Sung et al., 2001). The mechanisms of α-synuclein propagation are unclear; however, alpha-synuclein seeding and propagation is considered a crucial process in PD development (Borghammer, 2017). Interesting, a recent study show that alpha-synuclein can be propagated by the traveling of lysosome vesicles along tunneling inter-cellular nanotubules from cell to another cell (Abounit et al., 2016).

Rab5 is a multifunctional protein that regulates the first steps in endocytic pathways and contributing to anchoring, trafficking, fusion of endosomal membranes, and autophagy-mediated recycling (Olchowik and Miaczynska, 2009). In addition, mutated Rab5 leads to an accumulation of enlarged early and late endosomes/ phagosomes and defects in the regulation of endosome/phagosome trafficking to lysosomes, which involves Rab7. After vesicle maturation, Rab7 coordinates the fusion of late endosomes with autophagosomes and LC3 requirement. Together, these findings indicate that Rab5 is involved in the formation and transportation of immature endosomes/phagosomes and LC3 signaling, thereby contributing to the first steps of autophagy. Rab5, LC3, and Miro have unique roles in endocytosis and trafficking of early endosomes and in autophagy and in the dynamics of mitochondria and ER (Girard et al., 2014; Wang et al., 2016; Wegner et al., 2010). However, α-synuclein toxicity related to trafficking and Rabs, Miro, or LC3 have not been elucidated.

Investigations of intracellular trafficking and autophagy dysfunction in neurological disorders have revealed that degradation via lysosomes is crucial to balanced axonal vesicles and lysosome trafficking. Furthermore, intracellular trafficking impairments lead to the accumulation of lysosome vesicles causng axonal swelling and neurite dystrophy. Other studies have shown that endocytic pathway alterations result in

accumulation of endolysosomes (endosomes fused to lysosomes), thereby impeding autophagy and resulting in α-synuclein accumulation.

Conclusions

Death of DA neurons during PD is a complex and multifactorial process. Aggregates and oligomers are associated with cell death, but the mechanisms of α-synuclein toxicity remain unclear. Investigations concerning the toxicity of alpha-synuclein indicate that disturbances of the ubiquitin-proteasome system and the function of lysosomes also are involved. Moreover, concomitant impairment of mitochondrial function generates oxidative stress, which produces excessive ROS and subsequent neurotoxic effects. Interactions between mitochondria and the ER are important for maintaining homeostasis in these organelles and, impaired interactions can also trigger cell death. Furthermore, α-synuclein accumulation and mitochondrial dysfunctions are major contributors to trafficking impairments, which further contribute to DA cell death. Overall, the current literature describes several contributors to the pathology of PD, but a comprehensive model of pathogenesis and order of effects has not been established yet.

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CHAPTER 3

EXPRESSION OF ANTEROGRADE AND RETROGRADE MOTOR PROTEINS AND MITOCHONDRIAL MOBILITY IN BRAIN AREAS EXPOSED TO ROTENONE

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Abstract

Parkinson´s disease (PD) is marked by the death of dopaminergic (DA) neurons located in the substantia nigra. The molecular mechanisms underlying this cell death in familial and sporadic PD are yet unclear, but it has become evident that in both forms of PD mitochondrial dysfunction and disturbances in mitochondrial mobility may play a major role. Rotenone is a pesticide epidemiologically linked to the development of sporadic PD. Its toxic, neurodegenerative effect in DA neurons is due to its high affinity inhibition of mitochondrial NADH dehydrogenase within complex I of the respiratory chain. Apart from that, rotenone has also been shown to alter microtubules dynamics by causing centrosome disorganization, microtubule depolymerization and destabilization, all affecting proper mitochondrial mobility. It is yet unknown whether rotenone also affects the function and expression of motor proteins essential for anterograde and retrograde mitochondrial trafficking. In the present study, we exposed cultured postnatal rat DA neurons to low concentrations of rotenone and evaluated mitochondrial mobility as well as protein expression of KIF1B and KIF5 (molecular motors for neuronal mitochondrial anterograde traffic) and of dynein c1h1, dynactin and syntaphilin (motor proteins involved in mitochondrial retrograde transport and anchoring). We showed that after exposure of rotenone for 48h at 0.5nM the expression of KIF1B and KIF5 significantly increased. However, after exposure at 0.1 or 0.3nM of rotenone KIF5 expression decreased. Interestingly, the expression of KIF1B decreased after exposure at 0.3nM of rotenone for 24h, revealing that the time of rotenone exposure could lead to different changes in motor proteins expression. Mitochondrial mobility decreased after exposure at 0.1nM and 0.5nM of rotenone. The expression of dynein decreased while dynactin expression increased after exposure at 0.3nM of rotenone. After exposure at 0.5nM the expression of dynein increased while dynactin decreased. The expression of syntaphilin isoform of 70kDa decreased after exposure of rotenone at 0.3 or 0.5nM, while the expression of isoform of 65kDa increased after exposure of rotenone at 0.3nM for 24h. Our results suggest that the disturbance in mitochondrial mobility due to rotenone exposure may not only be ascribed to a direct effect on mitochondria and microtubule assembly but also on modulation of the expression of motor proteins involved in mitochondrial trafficking. These findings may provide new insights in the cellular processes that lead to mitochondrial dysmobility-related neurodegeneration.

INTRODUCTION

Parkinson´s disease (PD) is characterized by the neurodegeneration of dopaminergic neurons located in the pars compacta of the substantia nigra. The molecular mechanisms underlying the death of these dopaminergic (DA) neurons are still unclear. They may be different for the different types of PD, of which about 10% is considered familial or genetic and 90% sporadic or idiopathic. Among the mutated genes in the familial forms of PD, the alpha-synuclein (SNCA) gene is most prominent (Olanow and Brundin, 2013). These mutations can lead to alpha-synuclein overexpression, in case of SNCA triplication, or the formation of aberrant alpha-synuclein molecules. Alpha-synuclein is a major constituent in the Lewy bodies, characteristic for affected DA neurons; the formation of alpha-synuclein aggregates in DA neurons carrying SNCA mutations is thought to induce neurodegeneration by directly frustrating normal protein homeostasis and increasing cell stress and indirectly (?) by disturbing mitochondrial trafficking and normal mitochondria function.

The link between PD and mitochondrial dysfunction was established after PD developed in young addicts using self-made heroin contaminated with the substance 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Langston and Ballard, 1983). MPTP is taken up by glia cells, converted into MPP+ by monoamine oxidase-B, and subsequently taken up by dopamine transporters of DA neurons. The mechanism by which MPP+ exerts its effects is attributed to interference with complex I of the electron transport chain in mitochondria (Langston and Ballard, 1983). This leads to the production of reactive oxygen species (ROS), a drop in ATP production, and subsequent calcium toxicity. Many toxins causing mitochondrial respiratory chain inhibition have since been found to induce PD-like symptoms, such as the herbicide paraquat (Liang et al., 2013) and the pesticide rotenone (Sherer et al., 2003). Many pesticides are now epidemiologically linked to the development of sporadic PD and most are linked to dysfunction of mitochondrial respiratory complex I.

Rotenone is, as indicated above, a natural pesticide that acts with high affinity as a specific inhibitor of mitochondrial NADH dehydrogenase within complex I of the respiratory chain. Apart from that, rotenone has also been shown to alter microtubules dynamics, by causing centrosome disorganization (Diaz-Corrales et al., 2005),

microtubule depolymerization (Choi et al., 2011) and microtubule destabilization (Srivastava and Panda, 2007). It is well known that destabilization of microtubules cause deficit of anterograde and retrograde transport carried out by motor proteins such as kinesins and dynein, respectively (Hirokawa et al., 2009). Furthermore, the inhibition of kinesin-dependent transport impairs the axonal drive of vesicles, organelles, neurofilaments, and other cellular components (Stamer et al., 2002). The kinesin (KIF) superfamily of molecular motors play an essential role in the general intracellular anterograde movement (Hirokawa et al., 2010). KIF1B is involved in synaptic vesicles and mitochondria trafficking. Mitochondria transport is also mediated by KIF5 (Tanaka et al. 1998), which is expressed in three different neuronal isoforms: KIF5A, B, and C. KIF5A is required for anterograde transport of neurofilaments, as well as participate in the balance between anterograde and retrograde transport (Uchida et al., 2009).

Mitochondrial biogenesis and recycling are mainly performed in neuron body at the central nervous system, although it may occur in axons and dendrites of peripheral neurons at lower rates (Amiri and Hollenbeck, 2008). The organelle goes from neuronal periphery to cell body through retrograde trafficking carried out by dynein and dynactin complex (Hollenbeck and Saxton, 2005). Mitochondria also can stay anchored at sites of high energetic demand, through their association with the cytoplasmic protein syntaphilin (Kang et al., 2008). Moreover, the impairment of retrograde mitochondrial transport can impair the proper function of the organelle affecting also its biogenesis, which is critically involved in the formation of synapses and dendritic spines, as well as in apoptotic process and neurodegenerative diseases (Van Laar and Berman, 2009).

It is evident that rotenone exerts its neurodegenerative effect in DA neurons by directly inducing mitochondrial dysfunction (via inhibition of mitochondrial NADH dehydrogenase) and by disturbing mitochondrial mobility (via microtubule destabilization). It is as yet unknown whether these rotenone-induced effects on mitochondria mobility are accompanied by alterations in the expression of anterograde and retrograde motor proteins involved in mitochondria trafficking.

To examine this, we have exposed cultured rat postnatal DA neurons isolated from the substantia nigra to low concentrations of rotenone and analyzed mitochondrial mobility and the expression of KIF1B, KIF5, dynein, dynactin and syntaphilin in these

cells.

MATERIAL AND METHODS

All the procedures were performed in accordance with Institutional and International Guidelines for animal care and use (Demers et al., 2006), as well as respecting the Brazilian federal law 11794/08 for animal welfare. Special attention was taken to minimize the number and discomfort of the animals used in the present research.

Cell culture

Methodology employed for cell culture was a modification of the previously described protocol (Kivell et al., 2001). In brief, neonatal Lewis rats had their brains dissected out to access the substantia nigra. Blood and epithelial cells were removed from the areas of interest in sterile cold solution consisting of NaCl 120mM, KCl 5mM, KH2PO4 1.2mM, MgSO4 1.2mM, NaHCO3 25mM, glucose 13mM, and pH 7.2. Subsequently, tissues were cut into smaller pieces and incubated with 0.05 % of trypsin (Gibco) for 40 min at 37ºC followed by trypsin inhibitor (0.006 %, Gibco). Cells were mechanically dissociated using a Pasteur pipette, and the cell solution was centrifuged at 300 g for 5 min. The supernatant was discarded and cells were resuspended in Neurobasal A medium (Gibco) supplemented with 0.25 mM Glutamax (Gibco), 2 % B27 (Gibco), 0.25 nM L-Glutamine (Sigma), and 40 mg/L Gentamicin (Gibco). Cells were plated at the concentration of 1,800 cels/mm2 on nunclon (Nunc) dishes treated with poly-D-lysine. Cultures were kept in a humidified incubator with 5 % CO2 at 37ºC for 9 days. Culture medium was changed 3h after plating the cells and every three days.

All cultures presented 50% of neurons; tyrosine hydroxylase immunoreactive cells were also present at the ratio of 42% among the total number of neurons (23% of the total number of cells) in substantia nigra culture. Rotenone was prepared with DMSO at final concentration of 0.01% (stock solution of 1 mM) and diluted in culture medium applied to cell cultures, after 7 days of culturing in concentrations of 0.1, 0.3, and 0.5 nM for 48 h for dose response analysis. The cells were exposed to 0.3 nM of rotenone for 12, 24, and 48h for time-response analysis. Control cultures were exposed

to 0.01 % DMSO diluted in culture medium. These experimental conditions do not trigger cell death (Chaves et al., 2010). After rotenone exposure cells were then subjected to protein extraction for mitochondrial anterograde molecular motors analysis through Western blot.

Cell culture characterization

Cell cultures were washed in PBS, fixed in 4% paraformaldehyde for 10 minutes and permeabilized with PBS containing 0.2% Triton for 30 minutes both at room temperature. Unspecific binding sites were blocked with PBS containing 2% NGS (Vector Laboratories), 0.2% Triton and 4% bovine serum albumin (BSA, Sigma) for 30 minutes at room temperature. Cells from substantia nigra were incubated, independently, with mouse polyclonal antibodies against microtubule associated protein 2 (MAP2) (1/1 000, M4403, Sigma) and tyrosine hydroxylase (TH) (1/1 500, MAB138, Millipore) for 24 hours at 4°C, followed by incubation with anti-mouse immunoglobulin conjugated to FITC (Jackson, 1/120) for 45 minutes at room temperature protected from light. Culture dishes were mounted with mounting medium containing DAPI (4',6-diamidino-2-phenylindole, Vector Laboratories) to visualize cell nuclei. Immunolabeled cells were analyzed using a fluorescence microscope Axiophot 2 (Zeiss) equipped with Axio Cam MRm and appropriated filters using a 20× objective lens. Quantification was done by comparing images taken of 2 fields per culture plate, in the total of 3 plates, using filters to visualize the label generated by FITC and DAPI. Cell culture characterization was repeated twice.

Cell death analysis in cell cultures exposed to rotenone

Substantia nigra cell culture after exposure to rotenone were stained with trypan blue stain solution (Gibco), through of the addition of 10 µl of it in cell culture medium. Trypan blue stains in blue the cytoplasm of cells with damaged plasma membrane and does not stain live cells, allowing cell death analysis. Immediately after the addition of trypan blue, the cells were examined under a microscope (Olympus) using an objective lens of 40× and photographed to detect stained cells, this experiment was repeated twice.

Western Blot Analysis of Mitochondrial Molecular Motors

Cultured cells were homogenized in PBS, pH 7.4, containing 1 %NP40, 0.5 %sodium deoxycholate, 1 % SDS, 1 mM EDTA, 1 mM EGTA, and 1 % protease inhibitor cocktail (Sigma). After centrifugation at 14,000 rpm for 20 min, the resulting supernatant was fractionated by SDS-PAGE (10 lg of protein/lane) using a 12 % tris–HCl gel at 100 V for 1 h. Proteins were transferred to nitrocellulose membrane for 1 h at 100 V. Blots were incubated in blocking solution comprised of 5 % skim milk in TBS-T during 1 h at room temperature. Analysis of anterograde and retrograde molecular motors were made using antibodies against KIF1B (Santa Cruz, L-20, sc-18739), KIF5A+ B + C (Abcam, ab62104), dynein c1h1 (Santa Cruz, R-325, sc-9115, 1/200) dynactin (Santa Cruz, H-300, sc-11363, 1/400) and syntaphilin (Santa Cruz, H-250, sc-33824, 1/200) diluted 1/1000, 1/200 and 1/500, respectively, in blocking solution and incubated either at room temperature for 1h (KIF1B and retrograde molecular motors) or overnight at 4 ºC (KIF5A+B+C). Horseradish peroxidase-conjugated secondary antibody incubations were performed at room temperature for 1 h with anti-goat 1/2000 (Amersham) or anti-rabbit 1/10000 (Amersham). Development was done after 5-minute incubation with enhanced chemiluminescence reagent (Millipore) and exposure to appropriated films (Hyperfilm ECL, Amersham Biosciences). After development, blots were normalized by incubating with anti-beta-actin antibody 1/1,000 (Santa cruz, C4, sc-47778) during 1h at room temperature; horseradish peroxidase-conjugated secondary antibody was incubated for 1h also at room temperature and developed as previously described. Normalization was done by dividing the values corresponding to the bands relative to proteins of interest by beta-actin values. Films were digitalized and quantified using ImageJ software (National Institutes of Health, USA).

Mitochondria Labeling

After treatments, live cells were incubated with MitoTracker Green FM (Molecular Probes, USA) diluted in culture medium, at the final concentration of 30 nM, for 30 min at 37 ºC. This experimental condition does not affect mitochondrial membrane potential (Buckman et al., 2001). Cells were washed, after staining, three times with phosphate-buffered saline in the dark and immediately analyzed on an A1R Nikon confocal microscope using the 60x objective and the 490 nm laser. For each mobility test, a series of 30 fluorescent images was taken every 20s. Three different

fields, containing approximately 20,000 mitochondria each, were evaluated per dish. Movies containing stationary and moving particles were built using ImageJ. The difference tracker plugins (Babraham Institute, Cambrige, UK) were employed to evaluate the number of total moving and stationary particles per 1,000 pixels. Results were presented as percent of moving mitochondria. In order to analyze any possible change in mitochondria membrane potential after exposure to rotenone, cells from the different groups were incubated with MitoTracker Orange CMTMRos, which accumulates only in mitochondria with intact membrane potential. Labeling and analysis protocol was the same as previously described. Results were represented as total number of labeled mitochondria.

Statistical Analysis

Western blot results were analyzed by one-way ANOVA followed by Bonferroni post-test. GraphPad Prism (GraphPad Software Inc., version 4.00, CA) was the statistical software employed. A p value ≤ 0.05 was considered to indicate statistically significant differences. All data are expressed as percent of control±standard deviation (SD). The experiments were repeated three times, using three dishes each time (n = 3).

RESULTS

Cell culture characterization

Quantification of neurons using MAP2 labeling in cell cultures showed that 59% of these cells express MAP2 being considered as neurons. Analysis of tyrosine hydroxylase expression in cell cultures demonstrated that 42% of these cells in culture are dopaminergic (Fig. 1B). Results demonstrate the suitability of this cell culture method to study substantia nigra cell culture.

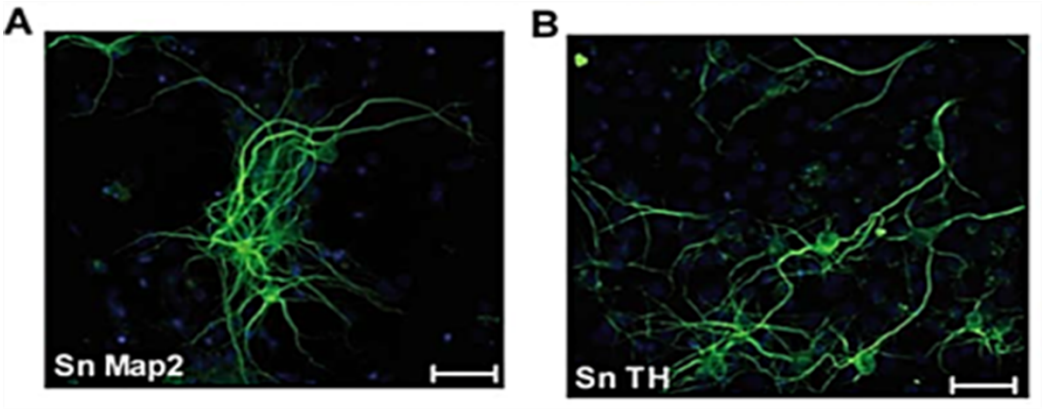

Figure 1: Cell cultures characterization. Illustrative digital images showing the immunoreactivity of microtubule associated protein 2 (MAP2) (A) and tyrosine hydroxylase (TH) immunoreactivity (B) in substantia nigra cultured cells. Scale bar is 50μm (Acta Neurobiol Exp, 2013).

Cell death analysis in cell cultures exposed to rotenone

Exposure to 0.5 nM of rotenone for 48 hours, the higher concentration applied to study the expression of proteins related to mitochondrial anterograde and retrograde trafficking, did not induce significant cell death (Fig. 2), similar to described in our previous study (Chaves et al. 2010). However, exposure to 10 nM of rotenone promoted a massive cell staining with trypan blue illustrating rotenone capability to induce cell death at high concentration (Fig. 2).

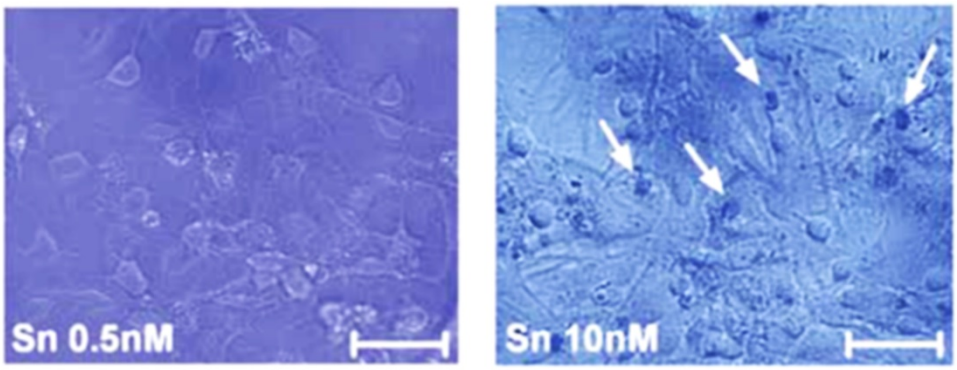

Figure 2: Cell death analysis in cell cultures exposed to rotenone. Illustrative digital images demonstrating tripan blue staining in cell cultures exposed to different concentrations of rotenone for 48 hours. Cells stained in blue (arrows) are under death process. Scale bar is 50 μm (Acta Neurobiol Exp, 2013).

Anterograde Trafficking and Mitochondrial Mobility Proteins are altered after rotenone exposure

KIF1B expression varied according to concentration and time of rotenone exposure. There was an increase of KIF1B expression after exposure of rotenone at 0.5 nM for 48h. Cultures exposed to 0.3 nM of rotenone by 24h showed a decrease of KIF1B expression (Fig. 3, upper panels). KIF5 expression was decreased after exposure to rotenone at lower concentrations than 0.5nM. However, 0.5 nM of rotenone applied over the cells during 48h promoted an increase in KIF5 expression (Fig. 3, middle panels). It is known that KIF1B and KIF5 work together in the mitochondrial anterograde trafficking. KIF5 is also important for the balance between anterograde and retrograde transport. Oxidative stress caused by rotenone can lead to cytoskeleton abnormalities. Alterations in KIF1B expression can disturb the formation of the complex with KIF5 and other proteins related to mitochondrial trafficking. Interesting, altered levels of KIF1B is linked to axonopathies and can be caused by abnormal cytoskeleton in PD (Falzone et al., 2009) and alterations in KIF1B expression also can lead to abnormalities in cytoskeleton and in intracellular trafficking (Gunawardena and Goldstein, 2001). Taken together, these results indicate that rotenone alters the levels of anterograde motor proteins and consequently anterograde mitochondrial trafficking could be altered. In order to analyzed mitochondrial mobility, cells were incubated with mitotracker green and the axonal regions of cells were identified and the total mitochondrial trafficking was analyzed (supplemental Fig.1). Mitochondrial trafficking decreased in cells after exposure to 0.1 and 0.5 nM of rotenone for 48 h (Fig. 3, lower panel). Rotenone leads to mitochondrial dysfunction and alters membrane potential. To investigate a number of mitochondria with membrane potential intact, cells were incubated with mitotracker orange. It was observed that the total number of mitochondria with intact membrane potential did not change after rotenone exposure (Fig. 3, lower panel).

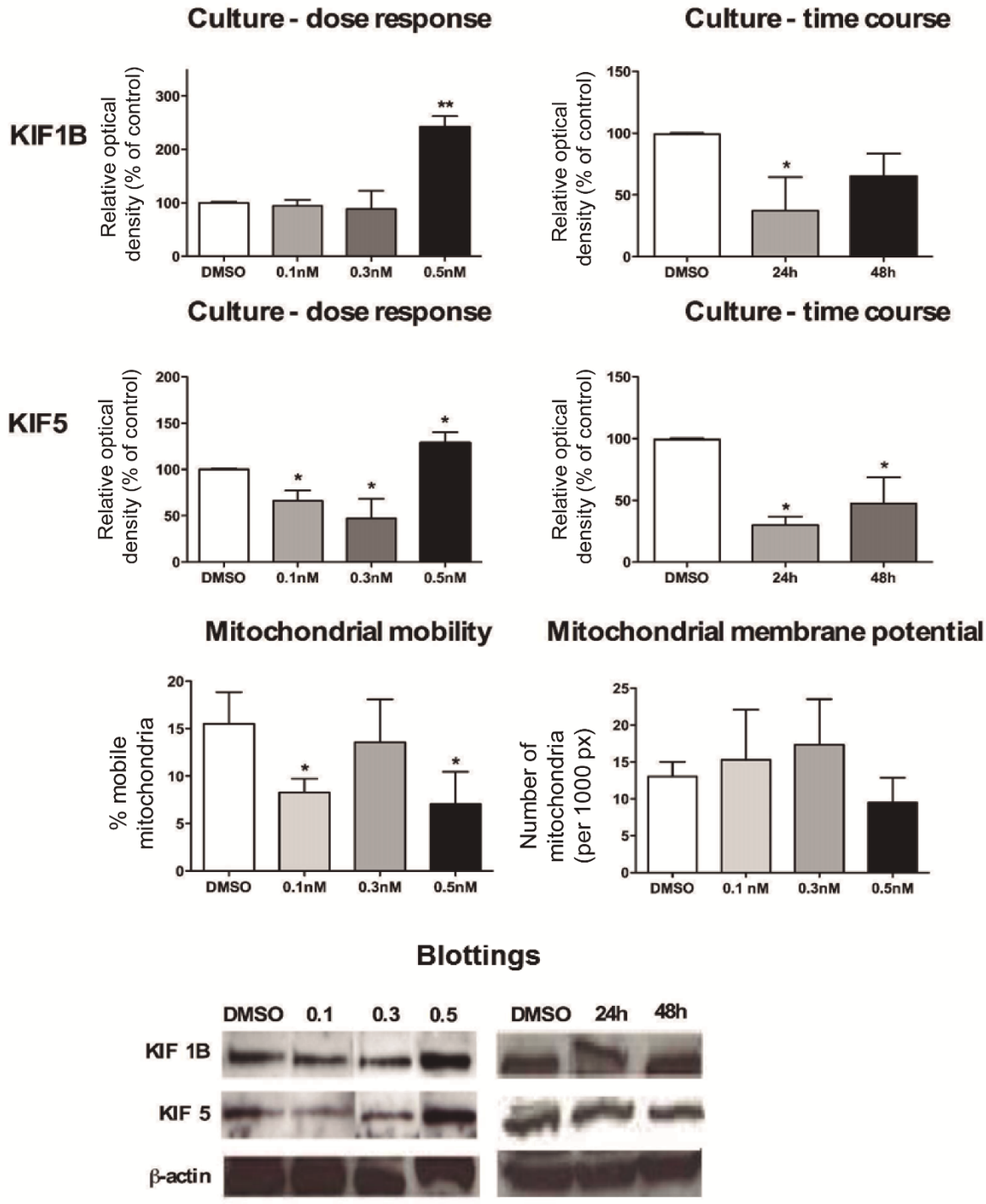

Figure 3: KIF1B and KIF5 protein expression in cell cultures exposed to DMSO (control) or rotenone at 0.1, 0.3, or 0.5 nM during 48 h (dose-dependent) or 0.3 nM during 24 or 48h (time-course). Mitochondrial mobility and number of mitochondria with intact membrane potential in cells from substantia nigra exposed to DMSO or rotenone at 0.1, 0.3, or 0.5 nM during 48 h. Data are expressed as percent or fold change relative to control (DMSO) ± SD as well as absolute number or percent of moving mitochondria per 1,000 pixels. Representative immunoblots are shown. One-way ANOVA (in vitro) following Tukey post-test was the statistical test employed. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$ compared with DMSO. n = 3 for cultures. Experiments were repeated 3 times (Molecular Neurobiology, 2012).

Rotenone exposure promoted a dual effect in dynein c1h1 and dynactin expression

It was observed a significant decrease of dynein c1h1 levels after exposure to 0.3nM of rotenone as compared to control cells, while 0.5nM of rotenone significantly increased dynein c1h1 expression as compared to 0.1 and 0.3nM (Fig.4 left upper panel). Dynactin protein expression decreased after exposure at 0.1nM and increased after exposure at 0.3nM of rotenone as compared to control cells (Fig.4 left middle panel). Time-course of mitochondria retrograde proteins responded accordingly to dose-response study, where it was observed a decrease in dynein c1h1 (Fig. 4 right upper panel) and an increase in dynactin after 0.3 nM of rotenone for 48 hours (Fig. 4 right middle panel). Representative immunoblots images of dose-response and time-response experiments are show in lower panel. These data suggest that rotenone changes in different ways motor proteins related to retrograde transport and probably may alter mitochondrial retrograde trafficking. As cited above, dynein and dynactin forms a complex to perfect coordinate mitochondrial retrograde transport. Considering that dynein and dynactin are connected for mitochondrial trafficking, the increased expression of dynactin may be a compensation of the decrease in dynein expression. Kimura and colleagues (2009) reported a similar response in dynactin expression after dynein depletion.

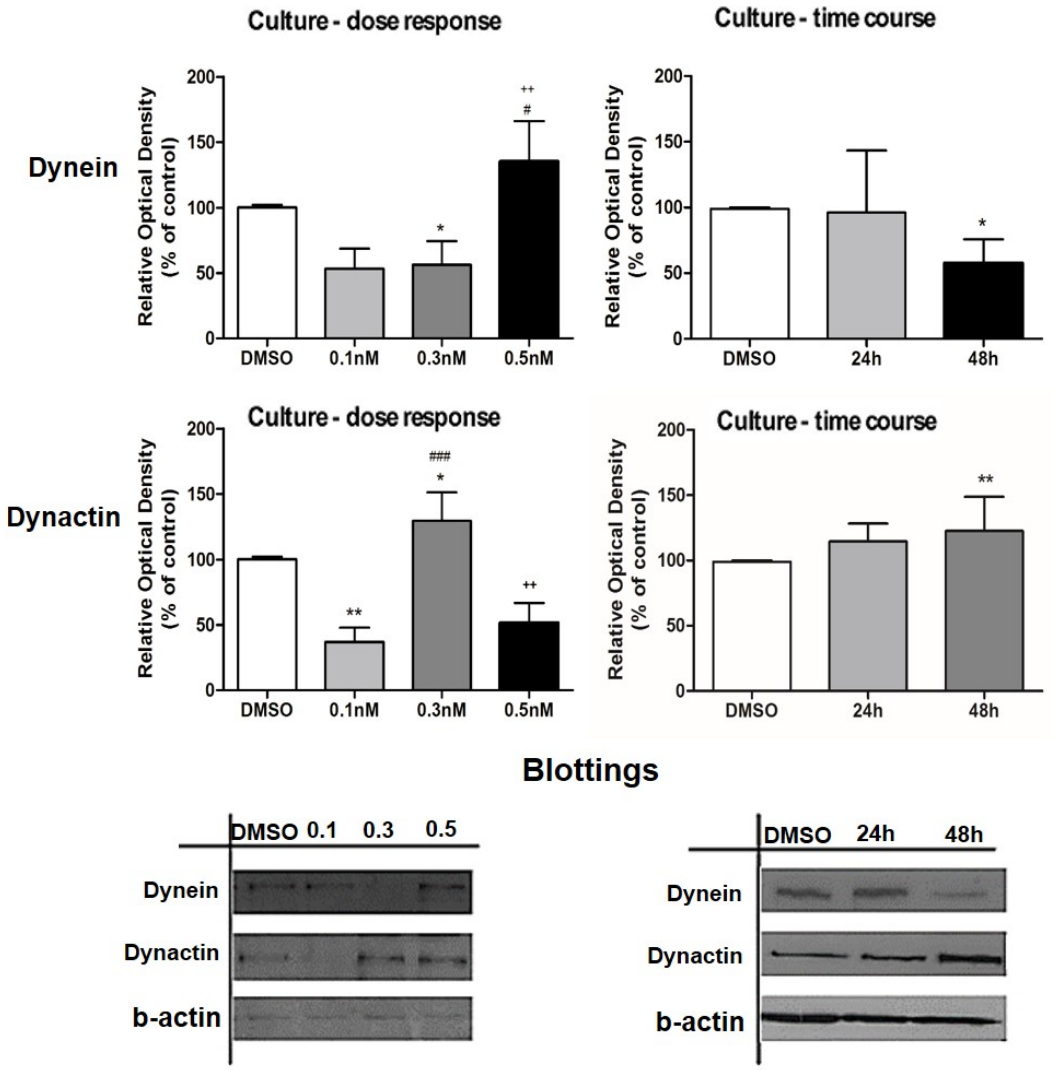

Figure 4: Dynein c1h1 and dynactin expression in cell culture exposed to rotenone. Dose-response and time-course of dynein c1h1 (left upper panel) and dynactin (left middle panel) protein expression in substantia nigra cell cultures exposed to 0.1, 0.3 or 0.5 nM of rotenone for 48 hours (dose-response); or 0.3 nM for 24 or 48 hours (time-course). Illustrative images of the pattern of bands corresponding to dose-response (left lower panel) and time-course (right lower panel) of dynein c1h1 and dynactin protein expression in substantia nigra cell cultures. Normalization was performed by beta-actin (43 kDa) signal. Data are shown as percent of control (DMSO) ± SD. * P<0.05; **P<0.01; ***P<0.001 as compared to DMSO, #P<0.05; ###P<0.001 as compared to 0.1 nM (dose-response), ++ P<0.01 as compared to 0.3 nM (dose-response), according to one-way ANOVA followed by Tukey post-test. Experiments were repeated twice, each run was performed in sample triplicates (Acta Neurobiol Exp, 2013).

Syntaphilin isoforms are differently regulated after rotenone exposure

Dose-response analysis of rotenone-exposed cells demonstrated a decrease in 70 kDa syntaphilin after 0.3 and 0.5 nM (Fig. 5 left upper panel), however, the isoform of

65 kDa did not change (Fig. 5 left middle panel). The time of rotenone exposure differently influenced syntaphilin isoforms expression, the 70 kDa isoform decreased after 24 and 48h of rotenone exposure (Fig. 5 right upper panel), however protein expression of the 65 kDa syntaphilin isoform increased after 24h of rotenone exposure as compared to DMSO control cells, returning to basal levels after 48h (Fig. 5 right middle panel). Fig. 5 lower panels show illustrative images of the pattern of bands of anti-syntaphilin in dose-response and time-course experiments. The dual syntaphilin response to rotenone exposure remains to be elucidated, however, the heterogeneity of response illustrates that rotenone exposure can alter syntaphilin expression and possibly mitochondrial anchoring in different ways depending upon organism and procedures of drug administration.

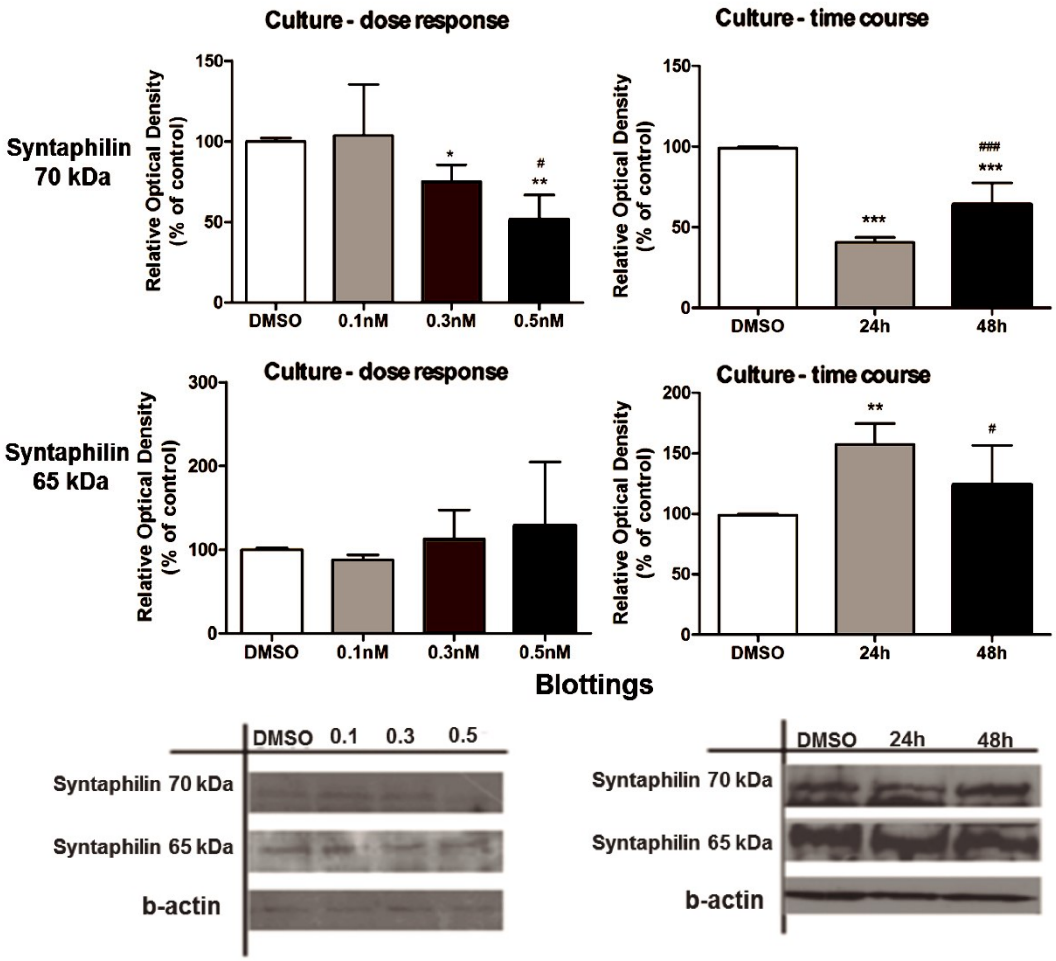

Figure 5: Syntaphilin expression in cell culture exposed to rotenone. Dose-response and time-course of syntaphilin isoforms of 70 (upper panels) and 65 kDa (middle panels) expression in substantia nigra cell cultures exposed to 0.1, 0.3 or 0.5 nM for 48 hours (dose-response); or 0.3 nM for 24 or 48 hours (time-course). Illustrative images of the pattern of bands corresponding to dose-response (left lower panel) and time-course (right lower panel) of Syntaphilin 70 and 65 kDa protein expression after rotenone exposure. Normalization was performed by beta-actin (43 kDa) signal. Data are shown as percent of control (DMSO) ± SD. *P<0.05; **P<0.01; ***P<0.001 as compared to control (DMSO), # P<0.05, ### P<0.001 as compared to 0.1 nM (dose-dependent) or 24 hours (time-course), according to one-way ANOVA followed by Tukey post-test to cell cultures. Experiments were repeated twice, each run was performed in sample triplicates (Acta Neurobiol Exp, 2013).

DISCUSSION

The present study showed for the first time, that exposure to low doses of rotenone lead to disturbance in mitochondrial trafficking by changing the expression of motor proteins related to anterograde or retrograde mitochondrial trafficking *in vitro*.

These findings suggest that change in mitochondrial trafficking, might be critical and one of the primary events for cell physiology impairment in the substantia nigra upon rotenone toxicity. The specific sensitivity of cells from substantia nigra to rotenone was confirmed by complemented quantification of KIF1B and KIF5 protein expression in somatosensory cortex as negative control after rotenone treatment in that area (unpublished data). Previous studies have reported that the rotenone capability to induce cell death in dopaminergic neurons (Phinney et al., 2006; Radad et al., 2008; Ren et al., 2005) and non-dopaminergic neurons as well as the capability to lead to microtubule depolymerizing (Ren and Feng, 2007; Srivastava and Panda, 2007), however, only at concentrations higher than 1nM, suggesting an effect in the motor proteins expression dependent of dose and independent of apoptotic processes.

In the present study, the exposure of rotenone modulated anterograde and retrograde motor proteins expression according to time and concentration administrated. These data indicate that the impairment of intracellular trafficking caused by changes in motor protein expression due the rotenone exposure is also time and dose-dependent in DA neurons.

Mitochondrial trafficking analysis showed that after exposure at 0.1 or 0.5nM of rotenone the trafficking decreased. It is known that either increase or decrease of mitochondrial mobility is implicated in neuron dysfunction (Sheng and Cai, 2012). If increase in mobility may be beneficial for biogenesis, on the other hand decrease in traffic may cause a deficit of mitochondria where it is required. Nonetheless, it was demonstrated a differential regulation of mitochondria mobility according to concentrations of rotenone administrated, and this may be of relevance to understand the vulnerability of DA from substantia nigra to rotenone.

Interesting to notice that the total mitochondrial trafficking seems to be regulated in a different way by the motor proteins. We observed that increased expression of KIF1B and dynein and decreased expression of syntaphilin led to decrease in mitochondrial trafficking, no matter what happened to KIF5 or dynactin. However, decreased expression of KIF5 and dynactin, in the absence of change in KIF1B, dynein or syntaphilin expression, led to decreased mitochondrial mobility. An interesting hypothesis is that KIF5, dynactin and syntaphilin seem to modulate positively

mitochondrial traffic, whereas, KIF1B and dynein seem to modulate negatively mitochondrial traffic. However, the real mechanisms under the interaction of mitochondria and motor proteins and how the trafficking is regulated is still unclear.

In conclusion, the present data suggest that the mechanisms of rotenone toxicity in DA involve changes in expression of motor proteins and mitochondrial trafficking and may be the primary events of neurodegenerative diseases associated to rotenone exposure. However, what direction, anterograde or retrograde trafficking, is affected first leading to specific mitochondrial dysfunction which is a characteristic of PD remains to be elucidated.

Acknowledgments

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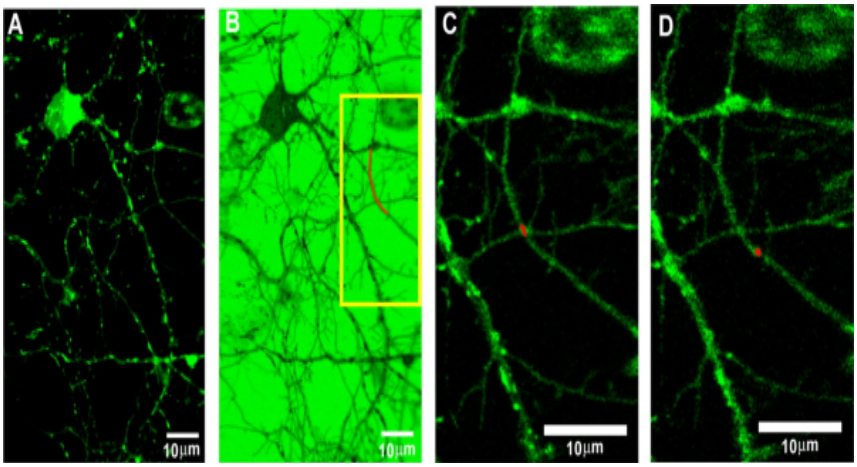
Supplementary information for chapter 3:

Supplementary figures:



Supplemental Figure 1: Photomicrographs of cultured cells from substantia nigra incubated with the probe mitotracker green. Identification of a cell and the presence of axons (A). Region chose to track mitochondrial mobility (B). Mitochondrial trafficking in time 0h (C) and in time after 20s (D). Mitochondria are highlighted in red (C and D).

CHAPTER 4

IMPAIRMENT OF MITOCHONDRIA DYNAMICS BY HUMAN A53T α-SYNUCLEIN AND RESCUE BY NAP (DAVUNETIDE) IN A CELL MODEL FOR PARKINSON´S DISEASE

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Abstract

The formation of oligomers and aggregates of overexpressed or mutant α-synuclein play a role in the degeneration of dopaminergic neurons in Parkinson's disease by causing dysfunction of mitochondria, reflected in their disturbed mobility and production of ROS. The mode of action and mechanisms underlying this mitochondrial impairment is still unclear. We have induced stable expression of wild-type, A30P or A53T α-synuclein in neuronally differentiated SH-SY5Y neuroblastoma cells and studied anterograde and retrograde mitochondrial trafficking in this cell model for Parkinson's disease. In contrast to wild-type and A30P, A53T α-synuclein significantly inhibited mitochondrial trafficking, at first retrogradely and in a later stage anterogradely. Accordingly, A53T α-synuclein also caused the highest increase in ROS production in the dysmobilized mitochondria in comparison to wild-type or A30P α-synuclein. Treatment with NAP, the eight amino acid peptide identified as the active component of activity-dependent neuroprotective protein (ADNP), completely annihilated the adverse effects of A53T on mitochondrial dynamics. Our results reveal that A53T α-synuclein (oligomers or aggregates) leads to the inhibition of mitochondrial trafficking, which can be rescued by NAP, suggesting the involvement of microtubule disruption in the pathophysiology of Parkinson's disease.

INTRODUCTION

Parkinson's disease (PD) is the most common neurodegenerative movement disorder affecting 1% of people above 65 years of age (Tanner, 1992). The pathology of PD is characterized by the degeneration of dopaminergic neurons in the substantia nigra. This neurodegeneration is thought to be associated with the formation of aggregates containing α-synuclein (Simcox et al., 2013) and with increased oxidative stress (Gruden et al., 2011; Qian et al., 2008; Simcox et al., 2013). The vast majority of PD patients have the sporadic form of this disease, which appears to be age related. A small percentage of PD is familiar and caused by specific mutations. The α-synuclein gene (*SNCA*) was the first gene definitely associated with familiar PD, and there are three known missense point mutations, A53T and A30P and E46 K, besides duplication and triplication of *SNCA*, that all lead to an early disease onset (Chartier-Harlin et al., 2004; Kruger et al., 1998; Polymeropoulos et al., 1997; Singleton et al., 2003). Various genome-wide association (GWAS) studies have shown that SNPs in *SNCA* (and *MAPT*) are also common risk factors for sporadic PD (Edwards et al., 2010; Lill et al., 2012) (Nalls et al., 2014).

The molecular mechanisms underlying the neurodegenerative effects of α-synuclein oligomers and aggregates seem to involve most prominently the mitochondria. Although direct damaging effects of α-synuclein oligomers and aggregates on mitochondria have been described, indirect effects on the processes of autophagy and trafficking of mitochondria may be involved as well. Alterations in intracellular degradation pathways, such as macro-autophagy, have been observed in many studies linking protein aggregation mechanisms with neurodegeneration (Victoria and Zurzolo, 2015). It has been demonstrated that overexpression of α-synuclein can lead to inhibition of autophagy and concomitant α-synuclein accumulation, whereas the knockdown of α-synuclein resulted in autophagy enhancement, suggesting that α-synuclein may have a regulatory role in autophagy (Winslow et al., 2010). The presence of damaged mitochondria by the direct action of (mutant) α-synuclein oligomers, on the contrary, appears to stimulate excessive mitophagy leading to mitochondrial fragmentation (Perfeito et al., 2014; Wang et al., 2012).

Normal mitochondrial turnover depends on a proper balance between

anterograde and retrograde trafficking (Arnold et al., 2011); in PD, this mitochondrial turnover appears to be impaired (Hunn et al., 2015; Simcox et al., 2013). In anterograde trafficking, mitochondria are transported from the soma to the axon up to the synaptic terminals; by retrograde trafficking, the mitochondria return to the cell soma for breakdown and re-entering the biogenesis cycle (Amiri and Hollenbeck, 2008). Retardation in anterograde transport can result in an abnormal cellular distribution of mitochondria and a decrease in ATP levels at the synapses (Cai et al., 2005); impairment of retrograde transportation leads to an accumulation of mitochondria in the synaptic terminal interfering with proper synapse formation and function (Van Laar and Berman, 2009).

Alpha-synuclein oligomers and aggregates seem to interfere directly with normal mitochondrial turnover (Celardo et al., 2014; Qian et al., 2008). Alterations in axonal transport and in the level of motor proteins have been observed in transgenic *Drosophila* co-expressing tau and α-synuclein, in postmortem brain tissue of sporadic PD patients, in animal and cellular models of sporadic PD and in rats overexpressing α-synuclein (Chaves et al., 2013; Chu et al., 2012; Melo et al., 2013; Roy and Jackson, 2014). Studies have shown the interaction between α-synuclein and tau (Credle et al., 2015; Magdalinou et al., 2015). In neurons, tau is essential for stabilizing microtubules and so for enabling proper motor transport (Wade-Martins, 2012). In case of overexpression of α-synuclein, tau is phosphorylated, leading to its loss of function and the subsequent impairment of trafficking (Credle et al., 2015; Magen et al., 2014). Esteves and collaborators demonstrated that α-synuclein oligomers are able to disrupt microtubules, leading to abnormal axonal trafficking and consequently mitochondrial dysfunction (Esteves et al., 2014). In particular, the α-synuclein gene mutation A53T is able to form oligomers and aggregates more easily and faster than other types of α-synuclein (Giasson et al., 2002). Accordingly, it was demonstrated that in particular A53T α-synuclein significantly reduced mitochondrial motility in cellular models for PD in which human A53T α-synuclein was expressed, i.e., in mouse hippocampal neurons and SH-SY5Y neuroblastoma cells (Xie and Chung, 2012) or in mouse cortical neurons (Li et al., 2013).

Activity-dependent neuroprotective protein (ADNP) is essential for brain

formation and provides neuroprotection throughout the entire adult brain; ADNP mRNA and protein expression responds to brain injury and a variety of cytotoxic insults. Structure-activity studies have identified a short eight amino acid peptide in ADNP, NAPVSIPQ (abbreviated to NAP) that appears to be responsible for neuroprotection (Bassan et al., 1999; Gozes, 2007; Zamostiano et al., 2001). Treatment with NAP has been shown to restore microtubule integrity and to rescue microtubules- dependent axonal trafficking, and, with that, mitochondrial function (Bassan et al., 1999; Esteves et al., 2014; Gozes, 2007; Zamostiano et al., 2001). NAP also contributed to functional recovery in mice overexpressing α-synuclein by reducing hyperphosphorylated tau levels (Magen et al., 2014).

In the present study, we aimed to analyze in more detail the effects of A30P or A53T α-synuclein on anterograde and retrograde mitochondrial trafficking in SH-SY5Y neuroblastoma cells in which we managed to induce a stable expression of wild-type, A30P or A53T α-synuclein. In addition, we have studied the effect of NAP treatment on the mitochondrial mobility and function in these cells.

MATERIAL AND METHODS

Cell culture

SH-SY5Y cells (passage 17), obtained from ATCC cell culture, were not used above passage 35 as these cells are reported to lose their neuronal phenotype after repeated passaging. Cells were maintained in DMEM (1×) supplemented with 15% FBS, 1% Pen/Strep, 100 mM Napyruvate and 2 mM Glutamax (DFCS) in tissue culture treated dishes or flasks. At 70–80% confluence, cells were passaged using trypsin/ EDTA (Lonza) following general cell culture procedures. Split ratios ranging from 1/20 to 1/40 were used to ensure similar densities among transgenic lines. To initiate differentiation into neuron-like cells, SH-SY5Y cells were plated at a density of 2 × 104 cells/ cm2 in wells pre-treated with poly-d-lysine (PDL). After 1 day, cells were exposed to 10 μM retinoic acid (Sigma) in DMEM (1×) supplemented with 10% FBS for 5 days, after which medium was replaced to DMEM 1× (High Glucose) containing 10 ng/ml BDNF (Peprotech), in order to promote the outgrowth of neural extensions.

Construction of viral vectors

Viral vectors empty or containing wild-type α-synuclein and mutant α-synuclein A30P or A53T (pENG1-3, a generous gift by Ellen Nollen) were constructed by PCR amplification (Phusion High-Fidelity DNA Polymerase, ThermoScientific) with overhangs for SpeI and NsiI. PCR amplicons and pSin- EF2-Nanog-Pur (Adgene plasmid 16578) were restricted using BcuI (SpeI, Thermo Scientific) and Mph1103I (NsiI, Thermo Scientific), and subsequently ligated using T4 ligase (ThermoScientific) after which the product was transformed in DH5α competent cells. PCR screening was performed to select positive colonies, which were checked by restriction analysis. Correct plasmids were sent for sequencing and transfected in HEK293T to validate expression. Vector constructs are shown in Supplemental Fig. S1.

SH-SY5Y viral transduction and transfection

Lentiviral particles were generated using a modified protocol based on the protocol of Trono et al. (http://tcf.epfl.ch/page-6764-en.html). Briefly, HEK-293T cells were transfected when cells were 70–80% confluent. A mixture containing 100 µl OPTIMEM (Gibco) 1.4 µg viral vector, 0.4 µg pMD2- VSV-G and 1 µg pCMV-D8.91, was supplemented with 6 µl FUGENE HD (Lonza) and incubated for 15 min at room temperature (RT) to generate transfection complexes. The next day medium was changed with 2 ml OPTIMEM, and viral particles were harvested between 36 and 48 h. Viral supernatant was collected and sterilized using a 0.45 µm filter (Nalgene), mixed with 10% DFCS in a 1:1 ratio and supplemented with polybrene (8 µg/ml). This mixture was added to a 6-well plate, and 1 × 105 cells were added to be transduced in suspension. The next day media were changed to DFCS 15%, and cells were placed on puromycin (2–4 µg/ml, Sigma) selection three days after transduction. Puromycin selection was continued during cell culture of the lines.

Characterization of SH-SY5Y by immunofluorescence

Transgenic cell lines cultured on PDL-coated coverslips were fixed in paraformaldehyde 4% for 20 min at RT and rinsed in PBS 3 times. Samples were permeabilized and blocked with PBS containing 0.1% Triton, 1% BSA and 5% normal goat serum for 60 min at RT. Samples were incubated with rabbit anti MAP2 (1:1000,

Abcam), mouse anti α-synuclein (1:500, Invitrogen) or rabbit anti TOM20 (1:1000 Santa Cruz FL-145) antibodies at RT for 1 h followed by incubation of fluorescent secondary antibodies (1:400) and Hoechst for 1 h at RT. Images were acquired using a Leica AF-6000 fluorescent microscope.

Mitochondria mobility

SH-SY5Y were transfected with pDsRed2-Mito (Clontech Cat. nr: 632421) in OPTIMEM (Gibco) using Lipofectamine 2000 (Invitrogen), following manufacturer's instructions. After 1 day of transfection, cells were exposed to 200 μg/ml of G418 for 2 weeks in order to select for cell lines containing the plasmid. SH-SY5Y were differentiated as described above, and mitochondrial mobility was measured in live cells after 4, 6 or 8 days of differentiation, using spinning disk confocal microscopy at 63× objective in a climate controlled chamber. The track was calculated by image comparison of the same field every 10 s for 20 min. Single-cell image stacks were analyzed using the ImageJ difference tracker plugin. Kymographs were generated using ImageJ (FIJI) Multi Kymograph plugin. The experiment was repeated three times independently.

ROS measurements

After 8 days of differentiation, ROS production was measured by incubating cells with CM-H2DCFDA probe (Invitrogen) at 10 μM for 1 min. Images were recorded using spinning disk confocal microscopy at a 63× objective and analyzed using ImageJ. We have used CM-H_2DCFDA [5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester], an improved, more stable, derivative of DCFDA, as a detector of ROS; this dye is not fluorescent when chemically reduced, but after cellular oxidation and removal of acetate groups by cellular esterases it becomes fluorescent. We measured fluorescence intensity of the transgenic neurons (i.e., expressing A53T, A30P or a surplus of WT α-synuclein) and the control (transfected with an empty vector).

Mitochondria morphology and connectivity measurement

After 8 days of differentiation, neurons were fixed and stained for mitochondria using TOM20 antibody. Mitochondria morphology and connectivity among fluorescent

mitochondria were observed and compared through stained neurons or neurons expressing pDsRed2-Mito. Total amount of neurons containing normal mitochondria morphology was analyzed and quantified.

Treatment with NAP

NAP (Santa Cruz, sc361778A) was dissolved in DMSO (Sigma Aldrich) at a stock concentration of 5 mM. Cells were treated with 5 nM NAP or vehicle, DMSO, for 24 h to evaluate rescuing of mitochondria trafficking.

Western blotting

At 8 days of differentiation, protein was extracted using protein lysis buffer. Protein lysates were sonicated and centrifuged at 13,000 rpm for 10 min. The supernatant was fractionated by SDS-PAGE (25 or 50 μg protein/lane) using a 12.5% tris–HCl gel at 100 V for 1.5 h. Proteins were transferred to immobilon membrane (FL-Millipore IPFL 00010) for 1 h at 100 V. Membranes were blocked using PBS with 0.5% Tween 20 and 5% milk for 30 min. The following antibodies were used directed against: β-actin (1:1000, 37 kDa, Abcam, #ab6276) and α-synuclein (1:1000, 14 kDa, Invitrogen, LB509). The blotted protein bands were visualized using Odyssey scanner (Li-Cor Biosciences, Lincoln, NE). Band density was quantified by computer-assisted image analysis software (ImageJ). For reliable quantification of the Western blot data, the defined methodology was followed as described by Taylor et al. (2013).

Real- time PCR

Total RNA and genomic DNA were collected using an AllPrep DNA/RNA/ Protein Mini Kit. CDNA was reverse transcribed from 1 μg of RNA using M-MLV Reverse Transcriptase. GAPDH mRNA and genomic GAPDH were used as reference for normalization. For quantitate real-time PCR, primers were acquired from Biolegio and reactions were run on a Biorad C1000 Touch thermal cycler and analyzed with Biorad CFX manager software.

Statistical analysis

Mitochondria trafficking, immunofluorescence, Western Blotting and real-time PCR results were analyzed by one- way or two- way ANOVA followed by Tukey's post hoc-test. Neurons containing normal mitochondria morphology were analyzed by

Student's t test. A p value ≤ 0.05 was considered significantly different, using GraphPad Prism software (GraphPad Software Inc., version 5.00, CA).

RESULTS

Characterization of differentiated, α- synuclein transgenic SH- SY5Y cells

After 4 days in vitro (DIV) in the presence of retinoic acid and BDNF, the transgenic SH-SY5Y cells (i.e., expressing A53T, A30P or a surplus of WT α-synuclein) and the control SH-SY5Y cells (transfected with an empty vector) differentiated into neuron-like cells and formed small neurite- like extensions; at 6 DIV, these cells started to express the neuronal marker MAP2 and continued to grow their neurite- like extensions reaching stable maturity at 8 DIV (Fig. 1a– c). No differences in the neuronal differentiation pattern were observed between the control cells and the ones expressing the α-synuclein variants (supplemental Fig. 2). Selective survival based on puromycin resistance resulted in a neuronally differentiated SH-SY5Y cell culture of which all cells expressed a high level either of A53T, A30P or WT α-synuclein; in the control SH-SY5Y cell cultures, expression of α-synuclein could not be detected through immunofluorescence (Fig. 1d). The expression of the α-synuclein variants in the differentiated transgenic and control SH- SY5Y cells was quantified at 8 DIV using qPCR (Fig. 1e). Results of qPCR showed that, although each of the transgenic cell lines contained a similar number of α-synuclein gene copies, transcriptional activity was lower in the cell lines containing mutant α-synuclein in comparison to those expressing wild-type α-synuclein, suggesting interference of these α-synuclein mutants with transcription, nuclear shuttling, or cytoplasmic mRNA processing.

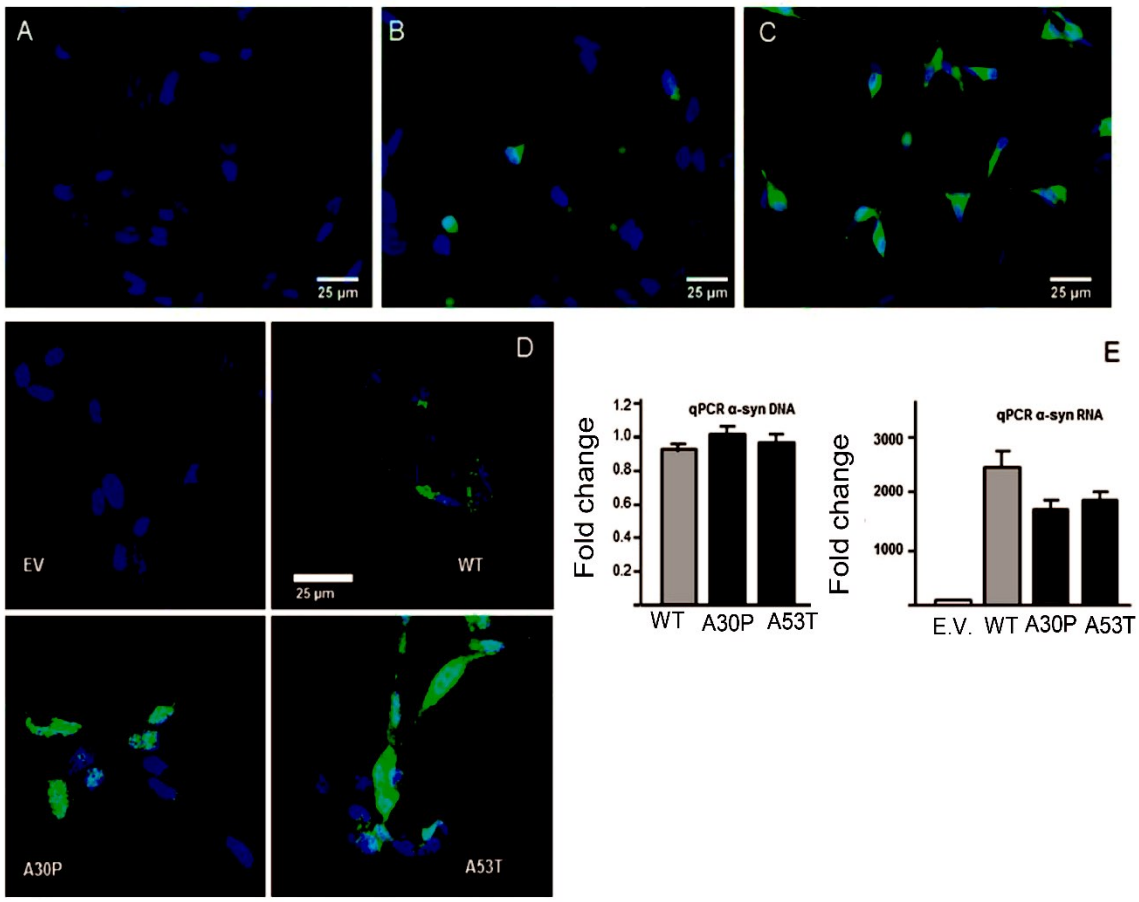

Figure 1: Characterization of differentiated, α-synuclein transgenic SH- SY5Y cells. Immunofluorescence shows a positive stain for microtubule-associated protein (MAP2) (*green*) at 4 days of differentiation (**A**), at 6 days of differentiation (**B**) and at 8 days of differentiation (**C**). Immunostaining for α-synuclein shows its expression in the α-synuclein transgenic SH-SY5Y cells (WT, A30P and A53T) but not in the control cells (transfected with an empty vector, EV) (**D**). *Blue* staining in **a**–**d** is Hoechst nuclear staining. Quantification of α-synuclein with q-PCR after 8 days of differentiation confirms the increased expression of α-synuclein related to WT (left panel) or E.V. cells (right panel) (E).

Analysis of α-synuclein linked to YFP corroborated the results of immunofluorescence, suggesting that A53T is more prone to aggregation since its pattern of labeling resembles that of small aggregates (Fig. 2). The expression of α-synuclein was quantified in Western blots demonstrating increased expression of A53T isoforms (Fig. 2). Our findings suggest a reduction in degradation, possibly due to the aggregated state of A53T α-synuclein.

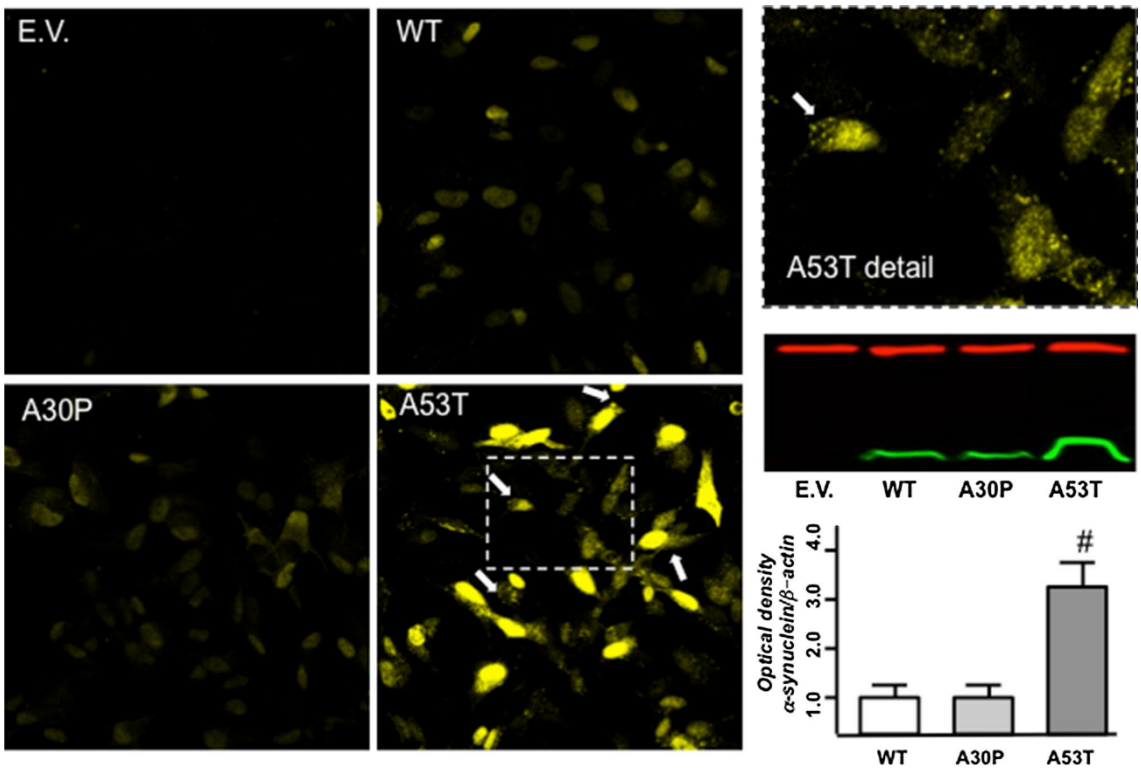

Figure 2: α-Synuclein expression. Photomicrographs show homogeneous α-synuclein staining in neurons expressing WT or A30P YFP- α-synuclein. Neurons expressing A53T YFP-α-synuclein showed puncta accumulation of the protein at soma and neurites (*arrows* in magnification of *boxed area*). Blottings of insoluble protein fraction (*red bands* = β-actin; *green bands* = α-synuclein) reveal significantly higher levels of A53T α-synuclein compared to WT and A30P α-synuclein. Experiments were repeated 3 times. #$p < 0.001$ as compared with WT according to one-way analysis of variance (ANOVA) followed by Tukey post-test.

Mitochondrial trafficking in differentiated, α-synuclein transgenic SH-SY5Y cells

We transfected the various neuronally differentiated SH-SY5Y cell cultures with the pDsRed2-Mito plasmid: approximately 50% of the cells showed red staining in the cytosol that appeared to be confined to mitochondria and completely co-localized with the green immunostaining by the mitochondria-specific TOM20 antibody (Fig. 3). The strong specific staining by pDsRed2-Mito allowed us to investigate mitochondrial mobility in the neurite-like extensions in the neuronally differentiated SH-SY5Y cells and the consequences of aberrant α-synuclein expression for mitochondrial trafficking. In order to examine if and when mitochondria traffic was impaired by α-synuclein in SH-SY5Y cell cultures, we analyzed mitochondria mobility in the different transgenic

and control SH-SY5Y cells at 4, 6 and 8 DIV in the presence of retinoic acid and BDNF representing different stages in differentiation. At 4 DIV, when the neuron-like cells were still immature and proper mitochondria trafficking was crucial for the outgrowth of the young “neurites,” a similar pattern of mitochondrial trafficking activity was observed in all the groups of SH-SY5Y-derived neuron-like cells. The first sign of disturbed mitochondrial mobility was observed at 6 DIV only in the A53T α-synuclein expressing SH-SY5Y cells where retrograde trafficking was significantly decreased in comparison to the control. Only later, at 8 DIV, also the anterograde trafficking of mitochondria was significantly decreased to almost 30%, again only, in the A53T α-synuclein expressing SH-SY5Y derived cells.

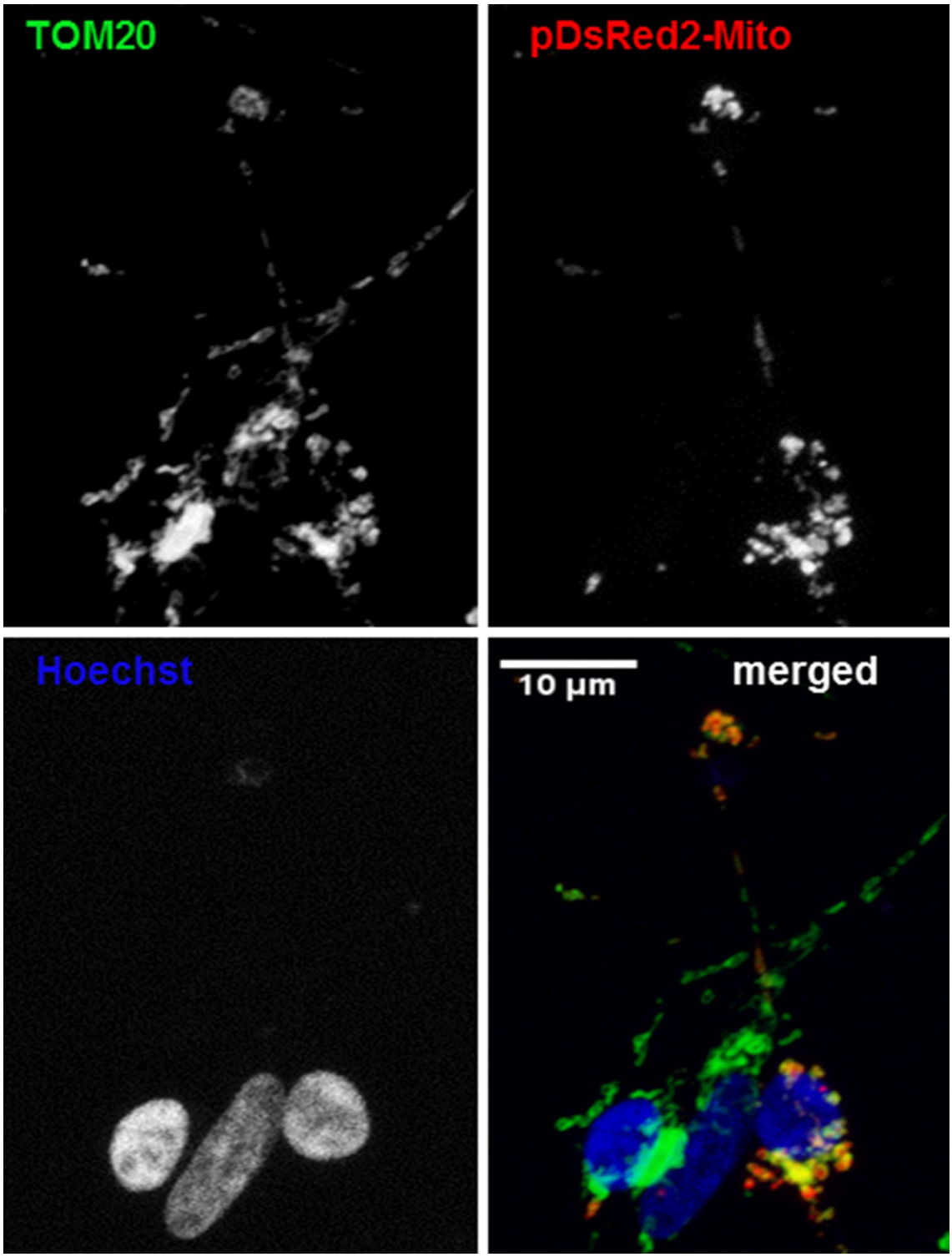

Figure 3: Mitochondrial labeling. Immunofluorescence imaging shows the co-localization of fluorescent staining for the mitochondria-specific TOM20 (*green*) and pDsRed2-Mito (*red*). In the merged picture, the nuclei are *blue* due to the Hoechst nuclear staining.

No changes were observed in the other transgenic SH-SY5Y cell lines or in the control cells (Fig. 4). So, Fig. 4 presents evidence for the presence of mitochondria

trafficking during differentiation and shows that the disturbance in mitochondria trafficking is specific for neuronal cells, since it does not occur in undifferentiated SH-SY5Y cells.

Effect of NAP on mitochondrial trafficking

To examine the mechanisms by which the A53T α-synuclein-induced impairment of mitochondrial trafficking in the neuronally differentiated SH-SY5Y cells, we treated the SH-SY5Y cell cultures with NAP since it has been shown to restore microtubule integrity (Esteves et al., 2014; Gozes, 2007). Adding NAP, indeed, appeared to completely restore retrograde as well as anterograde mitochondria trafficking in the A53T α-synuclein expressing SH- SY5Y derived cells (Fig. 4). Although both motor proteins, dynein and kinesin, have clear structural distinctions and wander across the microtubule surface with different speed, step sizes and in opposite directions, apparently the A53T α-synuclein-induced microtubules destruction, restorable by NAP, affects the interaction between the motor proteins and the microtubules in a similar mode.

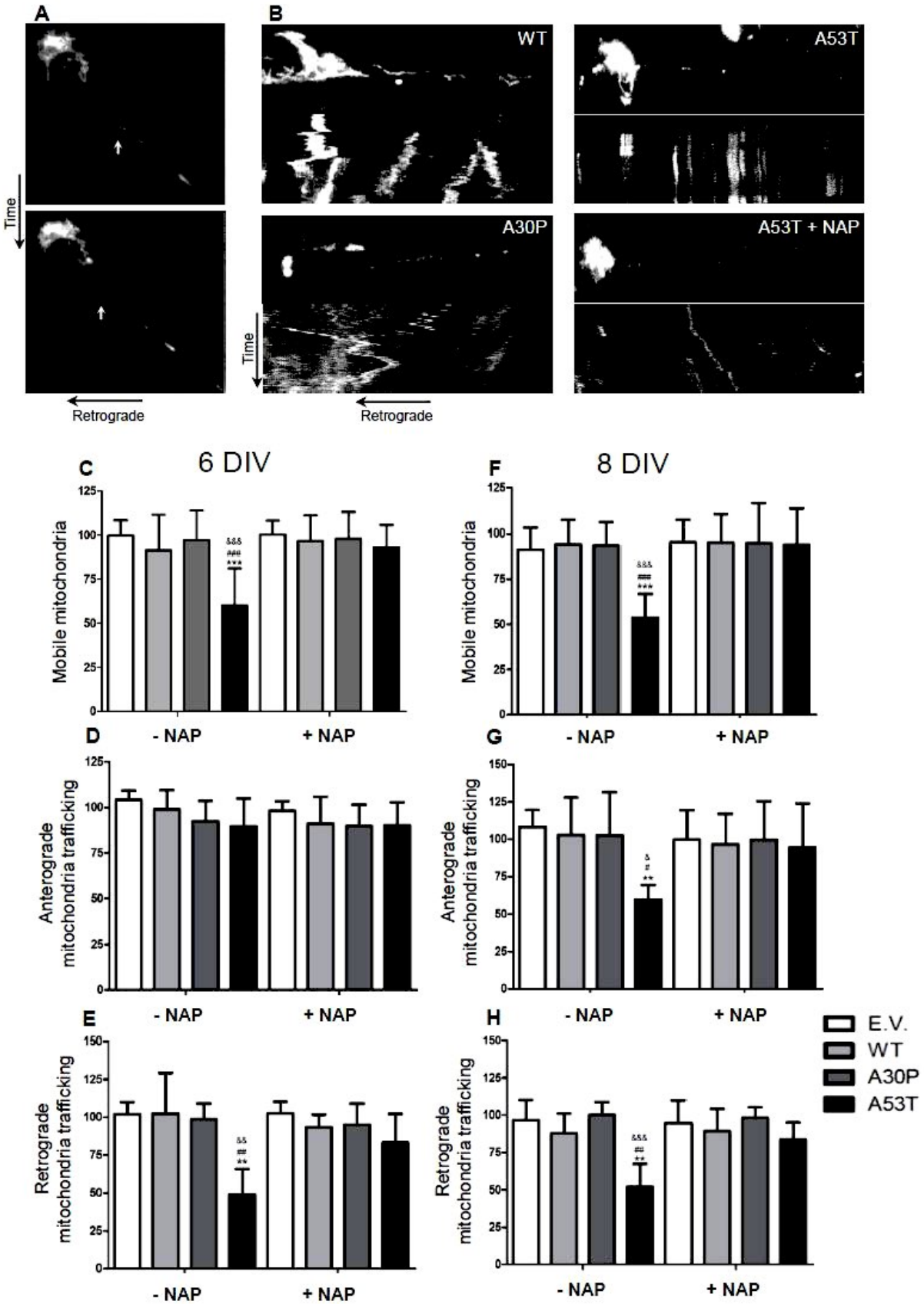

Figure 4: Mitochondria mobility analysis per 1000 pixels in SH-SY5Y. **A** Time-lapse recordings show the movements of mitochondria retrogradely in the neurite-like extension of a SH-SY5Y cell after 8 days of differentiation. **B** Kymographs represent mitochondria movement in neurons derived from SH-SY5Y cells after 6 days of

differentiation expressing either wild-type, A30P or A53T α-synuclein (in the absence and presence of NAP). Analysis of movement reveals that trafficking is decreased at 6 and 8 days of differentiation (**C**, **F**, respectively). Separate analysis of retrograde and anterograde trafficking shows unaltered anterograde trafficking but decreased retrograde trafficking at 6 days of differentiation (**D**, **E**); at 8 days of differentiation both anterograde and retrograde trafficking appears to be reduced (**G** and **H**, respectively). Neurons treated with NAP at 5 nM for 48 h show a rescued mitochondria trafficking at 6 and 8 days of differentiation (**C**, **E**–**H**). Data of moving mitochondria per 1000 pixels are expressed as percent relative to control (E.V.) ± SD. Two-way ANOVA following Bonferroni posttest were the statistical tests employed *$p \leq 0.05$ compared to control. #$p \leq 0.05$ compared to cells expressing WT. &$p \leq 0.05$, compared to cells expressing A30P α-synuclein. Data are expressed as mean of three independent experiments.

Effect of NAP on mitochondrial membrane potential and morphology

Impairment of mitochondrial dynamics due to the overexpression of α-synuclein or the expression of its mutants A30P and A53T may involve an increase in the production ROS and a disruption of the mitochondrial membrane potential. We found a significant increase in ROS levels in the α-synuclein expressing cells in comparison to the controls, with the highest level in the A53T mutant (Fig. 5a, c). Adding NAP to the culture, completely annihilated the ROS increase observed in all the α-synuclein expressing cell lines (Fig. 5b, c).

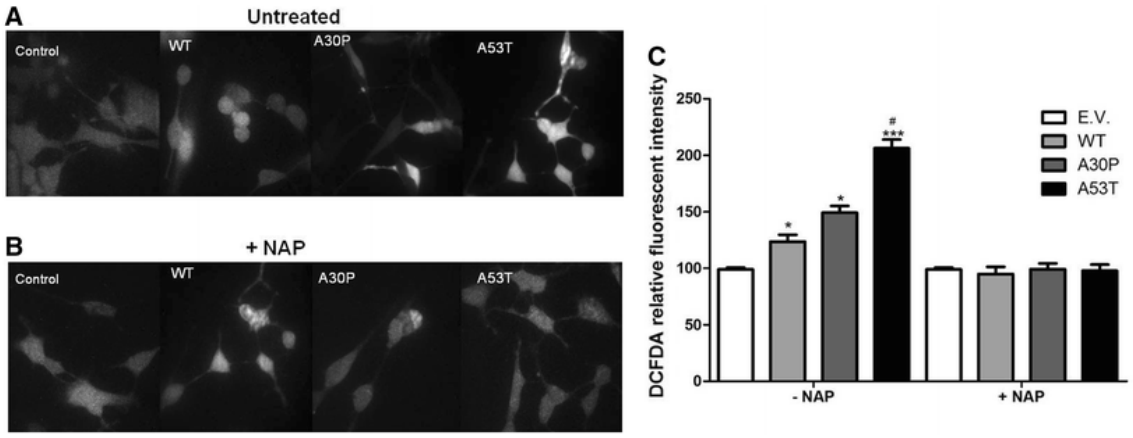

Figure 5: ROS production. Photomicrographs show neurons incubated with green fluorescent probe DCFDA untreated (**A**) or treated with NAP (**B**). Differentiated SH-SY5Y cells at 8 DIV expressing WT, A30P or A53T α-synuclein or controls (E.V. = empty vector) were incubated with CM-H2DCFDA, a fluorescent ROS detector, untreated (**A**) or treated with NAP (**B**). Fluorescence intensity measurements (**C**) show significantly higher levels of ROS compared to control (E.V.); treatment with NAP restored levels of ROS to basal levels of control cells. Data are expressed as percentage of control (E.V.) ± SD. Two- way ANOVA following Bonferroni posttest was the statistical test employed *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$ compared to control. #$p \leq 0.05$ compared to cells expressing WT. Data are expressed as mean of three independent experiments.

Living neuronal cells transfected with pDsRed2-Mito or those fixed and stained for TOM20 showed the same pattern of mitochondria morphology when expressing WT or A30P alpha-synuclein compared to control (transfected with empty vector). About 65% of neurons expressing A53T alpha-synuclein showed small spherical mitochondria clusters. After NAP treatment, only 20% of DA neurons expressing A53T alpha-synuclein showed fragmented mitochondria (Fig. 6).

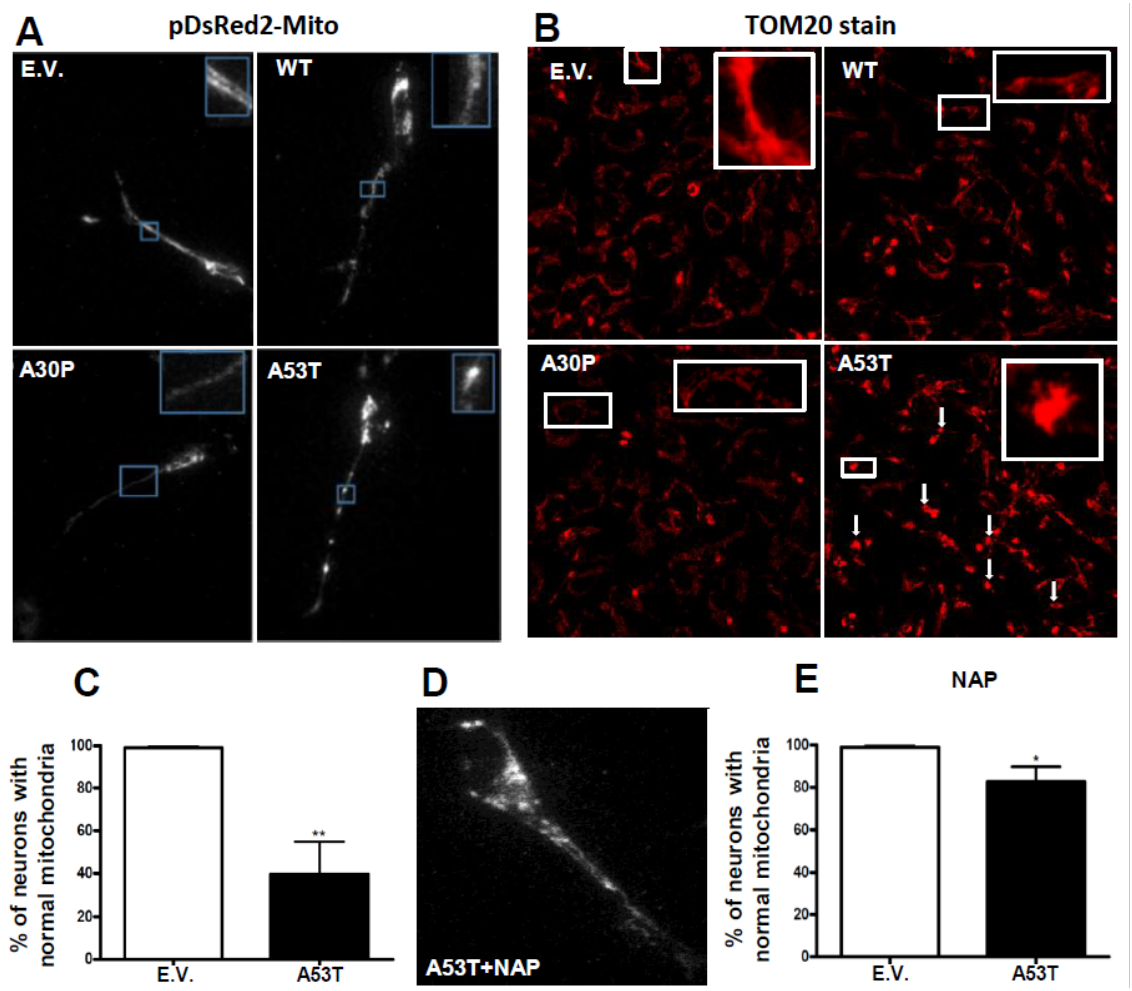

Figure 6: Mitochondria distribution and connectivity. Photomicrographs illustrate mitochondria distribution and connectivity in neurons transfected with pDsRed2-Mito (63× objective) (A) or in neurons stained for mitochondria using TOM20 antibody (20× objective) (**B**). Quantification of neurons with normal mitochondria distribution reveals a significant decrease after expression of A53T α-synuclein (**C**). Treatment with NAP increases the amount of neurons with normal mitochondria distribution and connectivity to 70% (**D** and **E**). Data are expressed as percent relative to control (EV). *$p < 0.05$ and **$p < 0.01$ compared with EV according to Student's t test. $n = 5$. Experiments were repeated three times.

DISCUSSION

Our studies show that, in contrast to wild-type and A30P α-synuclein, A53T α-synuclein significantly inhibited mitochondrial trafficking in the SH-SY5Y cell model for Parkinson's disease. Retrograde trafficking appears to be the first to be disturbed, followed in a later stage by anterograde trafficking. Accordingly, A53T α-synuclein also caused the highest increase in ROS production in these apparently demobilized and

fragmented mitochondria in comparison to wild-type or A30P α-synuclein. Treatment with the active peptide (NAP) of activity-dependent neuroprotective protein (ADNP) completely annihilated the adverse effects of A53T on mitochondrial dynamics.

Our findings regarding the effect of A53T α-synuclein on mitochondrial trafficking not only confirm but also extend previous data (Li et al., 2013; Xie and Chung, 2012) since we also studied the time frame of mitochondria trafficking disturbance: we wanted to determine which type of trafficking was disturbed first, anterograde or retrograde. Impairment of anterograde or retrograde trafficking can lead to different injuries in organelles in neurons. In our previous studies (Chaves et al., 2013; Melo et al., 2013), we revealed that the expression of proteins involved in anterograde or retrograde trafficking was differentially affected. It has been reported that disrupted anterograde mitochondria trafficking leads to fragmentation of mitochondria, abnormal mitochondria distribution and biogenesis, depletion of ATP and high ROS levels, resulting in cell death (Matenia et al., 2012), while disrupted retrograde trafficking leads to aging and swollen mitochondria, depletion of ATP, higher ROS levels and cell death (Morris and Hollenbeck, 1993). Although the outcome (cell death) is the same, determining the direction of trafficking that is impaired first enables elucidation of the actual mechanisms responsible for neuronal death; it may lead to a more specific therapeutical approach to prevent the phenotype of the disease. In the present study, we showed that A53T first impairs retrograde trafficking in neurons derived from the SH- SY5Y cells. In addition, we showed that NAP is able to recover mitochondria trafficking. The prominent effect on mitochondrial trafficking of A53T α-synuclein in comparison to A30P and wild- type α-synuclein most likely reflects the fact that A53T α-synuclein is more prone for oligomer or aggregate formation. It has been shown that the interaction of wild- type α-synuclein and α-synuclein variants with molecular motors, tubulin, and the microtubules-associated proteins, MAP2 and Tau, is stronger for oligomers than for monomers (Prots et al., 2013). There appears to be differential effects between seeds and oligomers on (Tau-promoted) microtubules assembly and on the microtubules gliding velocity across kinesin-coated surfaces (Prots et al., 2013). Due to the fact that NAP, a protein known to repair microtubules integrity, restores the A53T α-synuclein-induced impairment of mitochondrial trafficking, may

point to a disruption of microtubules assembly by A53T α-synuclein disturbing the interplay between microtubules and kinesin (Prots et al., 2013). The earliest effects of A53T α-synuclein was observed in the retrograde dynein-driven trafficking of mitochondria, indicating that also the dynein-microtubules interplay may be disturbed. The reason that retrograde mitochondrial trafficking is the first to be affected may be related to the lower velocity of retrograde transport in which even small disturbances leads to an actual stop in trafficking. Obviously, the machinery underlying mitochondrial trafficking along the microtubules in axons concerns a complex structure consisting, besides of kinesin and dynein, of Miro (also known as RhoT1/2) and Milton (Schwarz, 2013). Interactions between oligomers/ aggregates of α-synuclein and Miro and Milton are as yet unknown.

Although the best known action of NAP is upon microtubule stabilization, this peptide has also been reported to affect autophagy (Gozes, 2016) and oxidative stress (Greggio et al., 2011; Sharma et al., 2011). In fact, we observed a decrease in ROS content after NAP treatment, which may be either the cause or the consequence of altered mitochondria trafficking.

A wide variety of studies have shown that changes in mitochondrial trafficking are the earliest events to occur in cell models for Parkinson's disease with abnormal α-synuclein expression (Arduino et al., 2015; Coskun et al., 2012; Franco-Iborra et al., 2016; Keogh and Chinnery, 2015; Prots et al., 2013). The stressed, dysmobilized, mitochondria start to produce high levels of ROS, subsequently leading to other cytopathological processes. The annihilating effect of NAP on the ROS levels in our cell cultures expressing α-synuclein variants must be ascribed to the recovery of the mitochondrial motility by NAP (Esteves et al., 2014).

Summarizing, we propose that A53T α-synuclein leads to impairment of trafficking in SH-SY5Y cells, most likely by a disturbance of microtubule integrity. This in turn leads to impairment of mitochondrial turnover, particularly in distal regions of the cell. NAP treatment rescues mitochondria trafficking and so proper mitochondrial function and turnover. After 8 days of differentiation of the SH-SY5Y cells, we observed aggregates only in neurons expressing A53T alpha-synuclein. So, likely we have oligomers of WT, A30P and A53T alpha-synuclein after 6 days of differentiation

and of WT and A30P after 8 days of differentiation. As cited above, time to form oligomers of alpha-synuclein is dependent of its type, where A53T alpha-synuclein oligomerizes easier and faster than A30P alpha-synuclein, which in turn oligomerizes easier and faster than WT alpha-synuclein. Moreover, not only the levels of oligomers influence cellular damage, but also the type of oligomer is important for the type and amount of damage in neurons (Stefanovic et al., 2015). In view of this, our findings suggest that we have more oligomers of A53T alpha-synuclein and that they are more toxic than oligomers of A30P or WT alpha-synuclein. The increase in ROS production we observed may be related with lowered mitochondrial integrity at distal sites. We have shown that these effects can be rescued by using NAP, negating the effect of α-synuclein expression and leading to increased mitochondrial turnover. Our data implies mitochondrial trafficking as an important mechanism relating mitochondrial damage to protein occlusions observed in Parkinson's disease. Early impairment of retrograde transport would lead to accumulation of damaged mitochondria near the axon terminal, which has been proposed as an early event in clinical cases of Parkinson's disease (Cheng et al., 2010).

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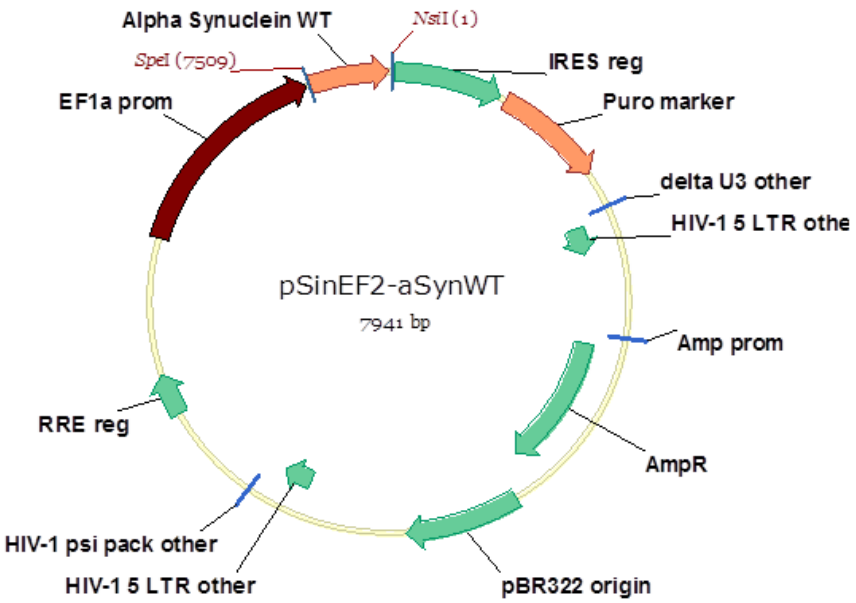
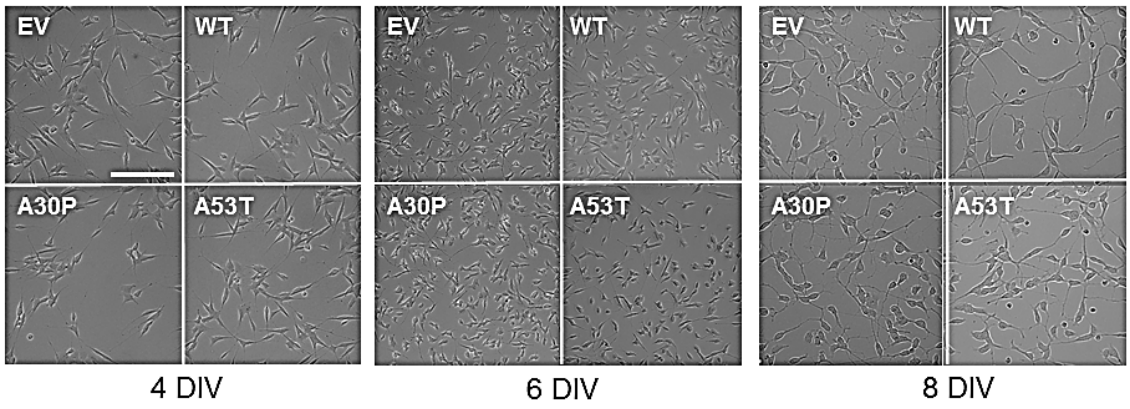
Supplementary information for chapter 4:

Supplementary figures:



Supplemental Figure 1: Example of the composition of the lentiviral vector used for the transfection of the α-synuclein (trans) genes.



Supplemental Figure 2: Neuronal-like differentiation of the various SH-SY5Y cell lines at 4, 6 and 8 days in vitro (DIV). No apparent differences in differentiation pattern were observed between the different cell lines. Phase-contrast microscopy, bar = 50 µm.

Supplemental Table 1. List of primers used.

Target	Fw. Primer	Rev. Primer
GAPDH	TTTCTATAAATTGAGCCCGCAGC	TACGACCAAATCCGTTGACTCC
GAPDH DNA	AACCTGCCAAATATGATGACATCA	AGCCCAGGATGCCTTTGAG
α-synuclein	AAGAGGGTGTTCTCTATGTAGGC	GCTCCTCCAACATTTGTCACTT
Plasmid α-synuclein	AACTAGTATGGATGTATTCATGAAAGGAC	AATGCATTTAGGCTTCAGGTTCGTAG
Seq. Primer EF1a	TCAAGCCTCAGACAGTGGTTC	

CHAPTER 5

MITOCHONDRIAL TRAFFICKING IMPAIRMENT IN DOPAMINERGIC NEURONS FROM PARKINSON´S PATIENT- DERIVED iPS CELLS

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Abstract

While it is widely recognized that Parkinson's disease (PD) is accompanied by mitochondrial dysfunction in dopaminergic (DA) neurons, the etiology of the disease remains elusive. Discovery of the implication of α-synuclein as the main constituent of Lewy bodies has driven research in the last decade, but the nature of the relationship between α-synuclein and mitochondrial dysfunction has not yet been elucidated. In this study, we use a patient-derived stem cell model of PD, comparing control cells with patient cells containing a triplication of or a mutation (A53T) in the SNCA gene. Using a long-term (90 days) culture approach, we analysed mitochondrial trafficking in cultured dopaminergic neurons. We demonstrated that mitochondrial trafficking is impaired in PD neurons when compared to control neurons, and mitochondria are frequently fragmented in patient-derived DA neurons. These results indicate that endogenous expression of α-synuclein in PD patient iPS-derived dopaminergic neurons results in mitochondrial pathology. Our results suggest that mitochondrial fragmentation and impaired trafficking are early events that contribute to mitochondrial dysfunction in PD.

INTRODUCTION

Parkinson's disease (PD) is the world's most common movement disorder and is characterized by rigidity, slowness of movement and resting tremors. The pathophysiological hallmark of PD is the occurrence of inclusion bodies in the brain named Lewy bodies. These cellular inclusions consist of abnormal protein aggregates and their occurrence is correlated to disease progression. Neuronal degeneration is most striking in the substantia nigra pars compacta in the ventral midbrain and dopaminergic (DA) cell death in this area is thought to contribute to most of the observed symptoms.

While research has been conducted for many years, the etiology of the disease remains poorly understood and treatment of PD merely addresses the symptoms. Experimental studies with substances that have been implied in causing specific dopaminergic degeneration, such as MPTP and pesticides like rotenone have elucidated at least part of the observed cell pathology, thus linking mitochondrial dysfunction to disease progression (Priyadarshi, Khuder et al. 2000, Jenner 2001). The discovery in 1997 of a dominant genetic form of PD (Polymeropoulos, Lavedan et al. 1997), linked to the SNCA gene, implicates α-synuclein in cell pathogenesis. As one of the major components in Lewy bodies, it has been shown that monomeric and oligomeric forms of α-synuclein are able to spread in a prion-like manner, leading to spreading of disease pathology (Braak, Ghebremedhin et al. 2004, Desplats, Lee et al. 2009). The link between α-synuclein and mitochondrial dysfunction remains poorly understood, and studies ranging from linking α-synuclein to mitochondrial permeability transition pore regulation Martin (Martin, Semenkow et al. 2014) to ER membrane stress have been performed (Smith, Jiang et al. 2005).

Most current understanding of cell pathogenesis in PD has been acquired through cell-models, genetics, animal studies and post mortem patient material. While these studies provided important insights in various cell pathological aspects, these approaches have their limitations. The scientific revolution of induced pluripotent stem cells (iPSCs) has led to the possibility of generating previously unobtainable cell populations from PD patients (Takahashi and Yamanaka 2006). Specifically, the generation of ventral midbrain dopaminergic neurons via iPSCs is bound to yield new understanding of PD and has already produced exciting new leads.

The link between mitochondrial dysfunction and α-synuclein has been further elucidated by assessing the vulnerability of PD iPSC-derived DA neurons to environmental toxins, showing a preferential vulnerability to maneb, paraquat and rotenone (Ryan, Dolatabadi et al. 2013). However, without using environmental toxins, hiPSC-derived DA neurons in culture conditions reveal little pathology, and have to be cultured for extended periods of time to observe pathological defects (Sanchez-Danes, Richaud-Patin et al. 2012).

It is hypothesized that early events in the synuclein cascade might compromise intracellular trafficking, leading to altered mitochondrial dynamics and development of early mitochondrial pathology (Cheng, Ulane et al. 2010, Hunn, Cragg et al. 2015). While there is evidence that points in this direction, data from iPSC-derived DA neurons, with long axonal processes have not yet been presented.

In this study, we have used dermal fibroblast-derived iPSCs from 2 autosomal dominant familial PD patients and a healthy gender and age-matched control to generate DA neurons. Our PD4 iPSC lines have a triplication of the SNCA locus (Parkinson Disease 4), and our PD1 iPSC lines have a reported mutation in the SNCA gene causing the missense variant A53T α-synuclein (Parkinson Disease 1). We show that (mutated) α-synuclein impairs mitochondrial trafficking in PD patient-derived iPSC-DA neurons cultured for 90 days in comparison to iPSC-DA neurons from healthy controls. Our findings provide evidence for a link between α-synuclein and early mitochondrial pathology in PD.

MATERIAL AND METHODS

Generation of hiPSCs

Human iPSCs were generated using a slightly modified protocol of Okita (Okita and Yamanaka 2011). Briefly, one million cells were nucleofected using an Amaxa nucleofector 1 using 0.85 µg pCXLE-hMLN, 0.85 µg pCXLE-hOCT3/4 and 1.3 µg pCXLE-hSK (Addgene #27079, #27076, #27078). Individual clones were picked and expanded, and checked for expression of pluripotency factors on regular basis (OCT3/4, SOX2, TRA1-60, NANOG, live alkaline phosphatase). Embryoid bodies (EBs) were

allowed to spontaneously differentiate in order to assess the pluripotent differentiation potential, followed by immunostaining for ectoderm (TUJ1), endoderm (GATA4) and mesoderm (DESMIN) (See supplemental figure S1). Geltrex® coated plates and Essential 8™ medium (Thermo Fisher) were used to maintain cells, while passaging was done using ReLeSR™ (Stemcell Technologies). All cells were maintained in a humidified incubator at 37°C with 5%CO_2 and used for experiments between passage 25 and 35.

Dopaminergic differentiation

Dopaminergic differentiation was achieved using a slightly adapted protocol from Kriks *et al. (Kriks, Shim et al. 2011)*. Briefly, cells were maintained to over 50% confluency after which medium was changed to 50% DMEM/F12 and 50% KSR medium (containing KnockOut™ DMEM and 15% KnockOut™ Serum Replacement, Thermo Fisher). Medium composition was gradually shifted to DMEM/F12 supplemented with N1 (Sigma) over 7 days, while the following small molecules were added; 10 μM SB-431542 (Stemcell Technologies, day 1-5), 100nM LDN-193189 (Stemcell Technologies, day 1-11), 3 μM Purmorphamine (Stemcell Technologies, day 3-7), 500 nM Smoothened agonist (Stemcell Technologies, day 3-7), 100 ng/ml SHH (Peprotech, day 3-8), 50 ng/ml FGF8 (Peprotech, day 3-8) and 2 μM CHIR99021 (Stemcell Technologies, day 5-13). At day 12, the medium was switched to Neurobasal A™ (NBA) medium (supplemented with SM1-Vit.A 50x (Stemcell Technologies), N1 250x (Sigma), BDNF (20 ng/ml, Peprotech), Ascorbic Acid (200 μM, Sigma), GDNF (20 ng/ml, Peprotech), dibutyryl cAMP (0.5 mM, Sigma), DAPT (10 μM, Stemcell Technologies), TGF-β3 (1 ng/ml, Peprotech) (BAGCDT) and 0,1% Pen/Strep (Lonza)) to start terminal neuronal induction. At day 20 of differentiation, cells were dissociated with Accutase (Sigma) and plated on Geltrex® coated tissue culture treated plates. To purify cultures, cultures were exposed to glucose deprivation and lactate (5mM) supplementation (GDLS) at 26DIV for 6 days (manuscript submitted). The resulting purified cultures were recovered in NBA for 6 days, dissociated with Accutase and passaged to either Geltrex® coated Nunc™ Lab-Tek™ II chamber slides™ or to Geltrex® coated borosilicate glass coverslips. Cells were maintained to 60 DIV, after which medium was changed to supplemented NBA without Pen/Strep until 90 DIV.

Calcium imaging recordings

Nunc™ Lab-Tek™ II chamber slides™ cultured neurons were incubated with 0.5 µM Fluo4-AM (Thermo Fisher, Cat No. F14217) and placed in an imaging chamber at 37°C with 5% CO2 and 95% humidity at 90 DIV. Image sequences were recorded using a Deltavision Elite live cell imaging system (GE Healthcare) equipped with PLAPON 60x oil, NA 1.42, WD 0.15 mm (Olympus) and a 15-bit EDGE/sCMOS camera (PCO) with GFP live filter wheel settings. Image sequences were recorded for 2 min intervals at 0.5 s per frame at 3.2% illumination intensity. Time series analysis was done using ImageJ (1.49s), plotting y-axis profiles for regions of interest (ROIs). Neuronal calcium events were defined as a sharp transient increase in fluorescence intensity (Fluo-4 AM, dF/F >5%, fast rise, slower decay). Images are presented using Green Fire Blue lookup tables.

Immunocytochemistry

Borosilicate glass coverslips containing neuronal cells were fixed in paraformaldehyde 4% for 15 min at room temperature and stored at 4°C. Permeabilization and blocking were done in PBS containing 0.1% Triton, 1% BSA and 5% normal goat serum for ~60 min at RT. Primary antibody incubation was done overnight at 6°C, followed by three PBS washes (5 min each), after with a fluorescent conjugated secondary antibody and Hoechst 33258 (Sigma, 14530) were added. In the case of using MitoTracker® Orange, live cells were incubated prior to fixation, since the dye fluorescence is retained after fixation. Mowiol® 4-88 (Sigma) was used as mounting medium to attach coverglasses to a coverslip. Samples were stored at 4°C until further analysis. Confocal images were acquired using a Leica SP8 confocal microscope, equipped with an HC PLAPO CS2 63x oil lens, with NA 1.4. Other epifluorescence images were acquired using a Leica AF-6000 fluorescent microscope, using a PL FLUOTAR 20x/NA 0.4, Dry lens.

Mitochondrial trafficking using MitoTracker® Orange

At 90 DIV, cells were incubated with 50nM MitoTracker® Orange CMTMRos (Invitrogen) for 30 min, after which medium was changed to fresh NBA supplemented medium.

Live imaging was performed using a Deltavision Elite live cell imaging system (GE Healthcare) equipped with PLAPON 60x oil, NA 1.42, WD 0.15 mm (Olympus) and a 15-bit EDGE/sCMOS camera (PCO) with mCherry live filter wheel settings, using 1 % illumination intensity. During live imaging, cells were maintained in a humidified imaging chamber at 37°C with 5%CO_2. Data sets were recorded at 2.5 s intervals, with a total of 301 frames per recorded image sequence at 2024x2024 pixels.

Analysis using ImageJ plugin Difference tracker

Supplemental figureS3 A-C illustrates a simplified example of the method we have used for analysing mitochondrial trafficking images. The ImageJ plugin DifferenceTracker can compare time series images to their previous frames to determine if pixels have shifted. Using a frame offset value determines if pixels have moved over a number of frames.

Comparing figure S3C to figure S3B, the plugin is unable to detect changes in the red boxed pixel, since it still records a positive pixel value in this frame. However, when looking at figure S3A a difference is observed, and the pixel can be classified as moving. In our approach, we have used a frame offset of four. Apart from frame offset, we used a difference in pixel intensity of at least 20 to be observed in an 8-bit image (256 grey values) to classify the pixel as moving. This is correlated to total number of pixels present, as this corrects between cell densities between groups. This leads to the reported % of pixels moving (i.e. % moving mitochondria), and % of moving intensities (i.e. %moving mitochondria corrected for fluorescent signal).

Overlay images were generated using ImageJ software, with pseudocolor red for DifferenceTracker generated images.

Statistical analysis

All statistical tests were performed using Sigmaplot 13.0. Statistical significance was determined using one-way ANOVA followed by Tukey or Bonferroni post hoc test. A p-value < 0.05 was considered significant. Data are presented as the minimum to maximum whisker box plots or dot plots.

RESULTS

PD patient iPSCs DA differentiation

iPSCs were generated from three fibroblast samples (one age-matched control and two PD patients) and characterized using standardized procedures. Supplemental figure S1 shows the characterization of iPSCs during different stages of generation.

We successfully differentiated all iPS lines into neurons with tyrosine hydroxylase (TH) expression in 10-20% of cells at 60 days in vitro (DIV) (figure1A). TH-positive differentiated cells show robust expression of α-synuclein compared to non-neuronal cells, which can be recognized by a larger nucleus (figure 1B), but no differences were observed between lines. To remove non-neuronal cells from culture, we applied a metabolic selection procedure developed by our lab (figure 1C). We found similar efficiencies in dopaminergic differentiation in all the different cell lines after metabolic selection (figure 1D-F). Calcium imaging revealed the generation of spontaneous action potentials in PD1 derived neuronal cells at 90 DIV indicating that these neurons were viable and active at the time of mitochondrial trafficking analysis (see supplemental figure S2 and movie S2).

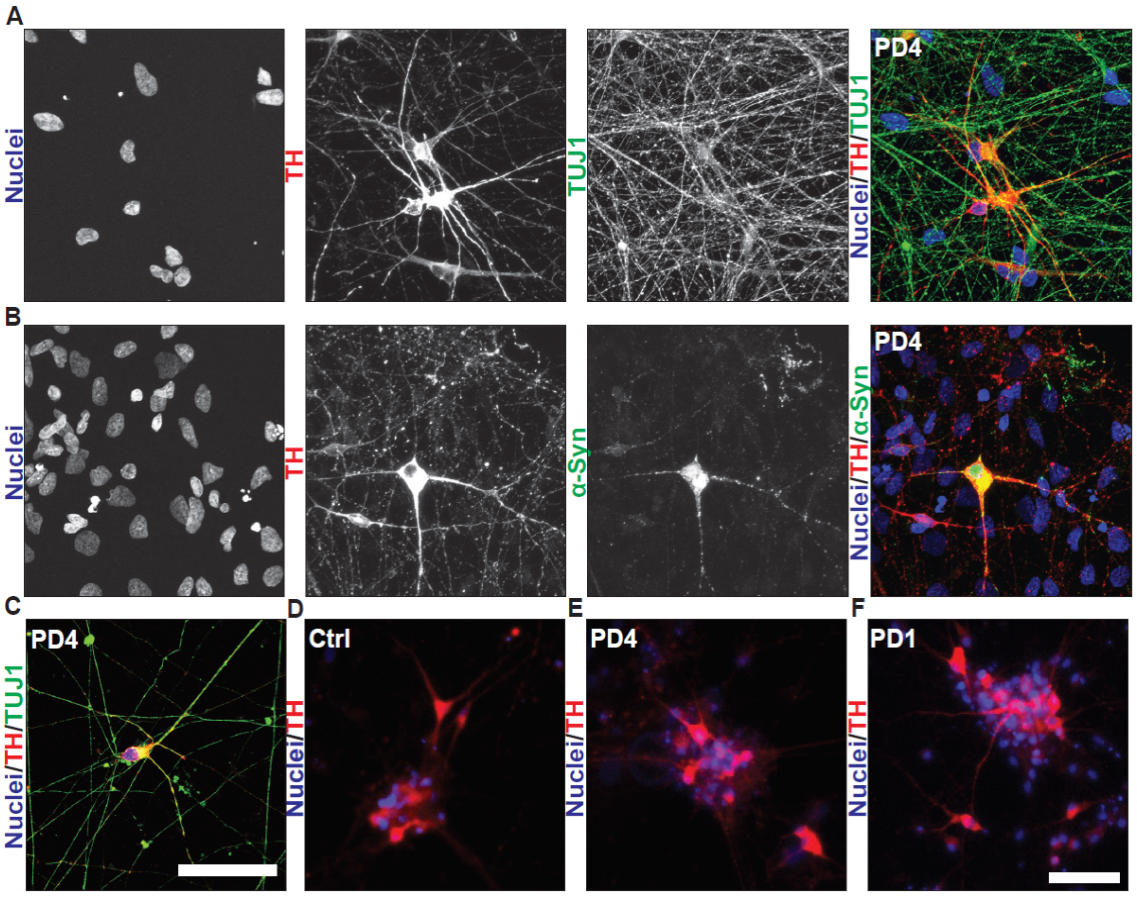

Figure 1: Patient hiPSCs can be differentiated into DA neurons (A) Differentiation of hiPSCs to neuronal cells at 60 DIV. Immunofluorescence imaging for TUJ1 (green), TH (red) and Hoechst (blue). (B) Neuronal cells display expression of α-synuclein (green), TH (red) and Hoechst (blue). (C) DA cultures can be purified to enrich for neuronal cells, staining for TUJ1 (green), TH (red) and Hoechst (blue) at 60 DIV. (D) Phase contrast image overlay with MitoTracker® Orange labeled neurons used in mitochondrial trafficking experiments at 90 DIV. (E-G) Comparison of purified cultures of Ctrl (E), PD4 (F) and PD1 (G) at 60DIV. Cells are labeled for TH (red) and Hoechst (blue). All bars represent 50 µm.

MitoTracker® Orange can be used as a specific marker for mitochondria in iPSC derived DA neurons

To validate the specificity of MitoTracker® Orange as a selective live dye for mitochondria in iPSC-derived dopaminergic neurons, a triple staining was performed using TOMM20, an outer membrane protein of mitochondria, TH and Hoechst33342. Fluorescence co-localization analysis was done using the ImageJ plugin Coloc2. The resulting Pearson's R^2 value of 0.89 points to a strong co-localization of TOMM20 with MitoTracker® Orange in whole image analysis, indicating that most mitochondria are stained with the live dye in dopaminergic neurons (figure 2A). Zooming in on axonal

structures (figure 2B) reveals a higher correlation with a Pearson's R^2 value of 0.91, further validating proper use of this live dye. On average, bleaching during 5 minutes of live imaging when using this dye was to less than 10% and was not significantly different between groups (see supplemental figure S3H).

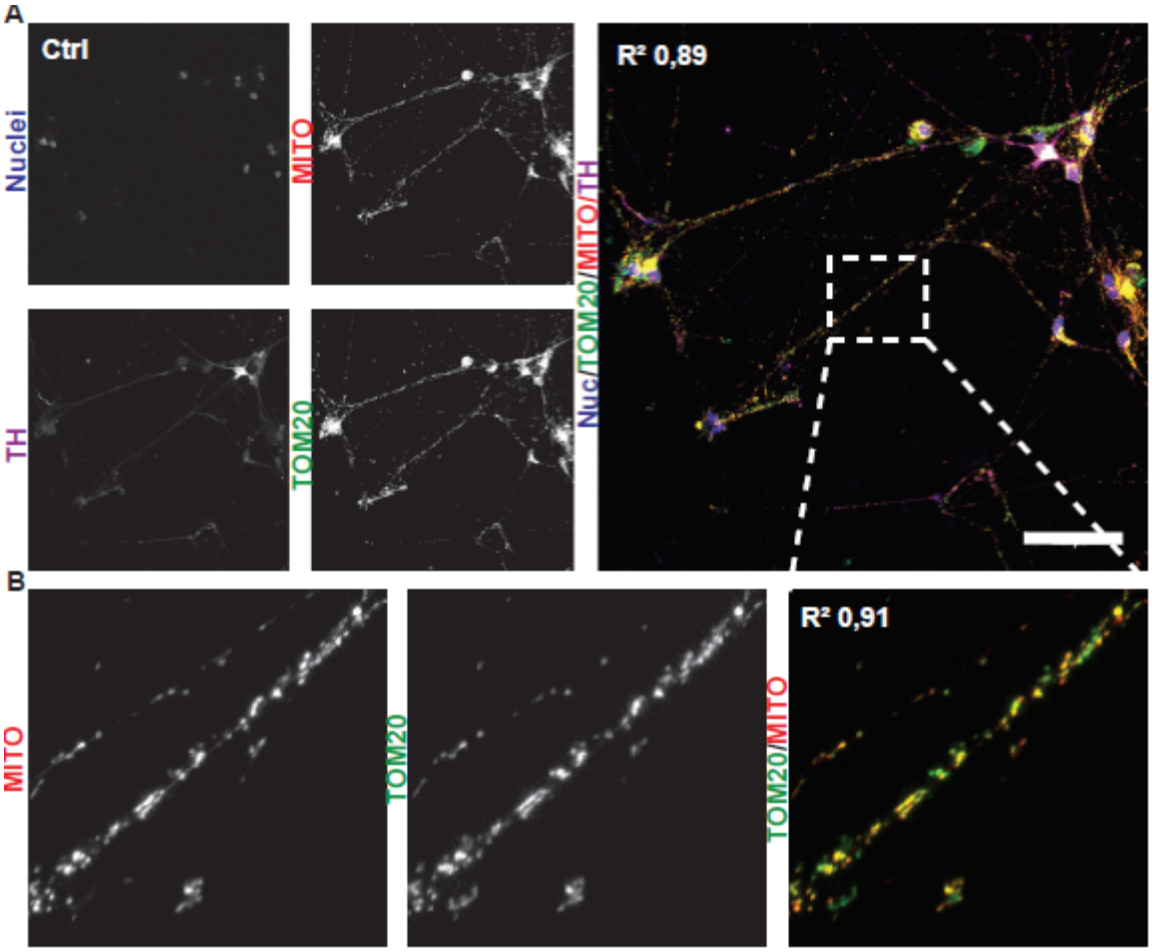

Figure 2: MitoTracker® Orange co-localizes with TOMM20 (A) Staining of MitoTracker® Orange labeled cells at 60DIV labeled with TH (magenta), MitoTracker® Orange (red), TOMM20 (green) and Hoechst (blue) (bars 50 µm). Pearson's correlation R2 for TOMM20 and MitoTracker® Orange in the whole image has a value of 0.89. (B) An axonal excerpt of figure A, for MitoTracker® Orange (red) and TOMM20 (green) has a Pearson's correlation R2 value of 0.91.

Analysis of mitochondrial trafficking in hiPSC-derived neurons

To evaluate the process of mitochondrial trafficking, fission and fusion we used an automated approach to identify trafficking of these organelles by employing the ImageJ plugin DifferenceTracker (see Materials & Methods). A simplified explanation, based on the authors' instructions is illustrated in supplemental figure S3. All trafficking events were recorded at 90 DIV, without use of neurotoxins, and under identical experimental conditions.

An analysis of an image of the control cell line at a window of 222.5 µm by

222.5 μm is depicted in figure 3B. In this image, moving pixels are pseudocolored in red, while stationary pixels are pseudocolored green. An excerpt of this image in figure 3A shows a more detailed image measuring 70 μm by 70 μm. A time series of 40 seconds of an even more detailed excerpt with dimensions 32.5 μm by 32.5 μm is presented in figure 3C. In this time series, we show a moving mitochondrion in red followed by the arrow over different frames, moving approximately 25 μm before moving out of frame. Calculation of the whole image with difference tracker on this excerpt revealed that 11.6 % of the pixels are moving over a total of 297 imaging frames. A movie is available as Movie S1.

The kymograph, corresponding to the line drawn in the last timeframe in figure 3C, provides an accurate representation of mitochondrial trafficking (figure 3D). However, we have chosen to show DifferenceTracker overlay images since these depict whole image trafficking events. Additional kymographs are presented as supplementary images (see figure S4).

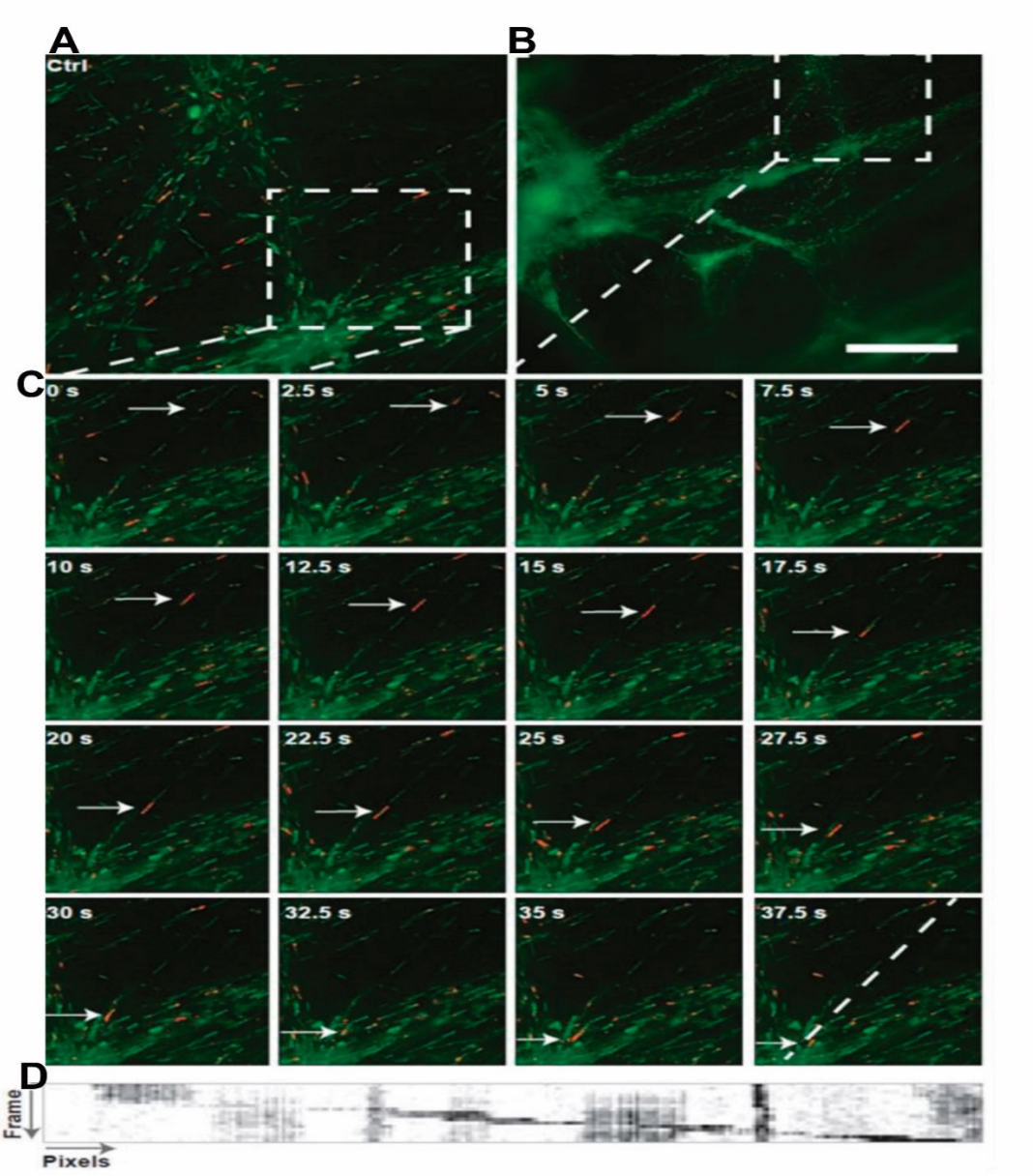

Figure 3: Difference tracker can be used to identify trafficking mitochondria (A) MitoTracker® Orange labeled culture analyzed using difference tracker. Red pseudo colors moving mitochondria, determined using the ImageJ plugin Difference Tracker, while green shows stationary mitochondria (bars 50 μm). (B) Shows an excerpt from (A) with enhanced detail, while (C) illustrates an even more enhanced excerpt with 16 consecutive frames to show movement of mitochondria. (D) Kymograph representation of the line drawn in (C), depicts mitochondrial trafficking over 16 frames.

Mitochondrial trafficking is impaired in PD neurons

Trafficking analysis was assessed using large frame images (as in figure 3) and images were excluded if cells moved out of focus or if cells moved, leading to false

positive trafficking events.

We determined the percentage of moving pixels in whole image analysis (figure 4A), and the percentage of moving pixel intensity, correlating to the percentage of moving mitochondria (figure 4B-D, see also corresponding kymographs in supplemental figure S4). Both graphs show a significant difference in trafficking of mitochondria in neurons between Ctrl and PD1 patient lines, but not between PD lines. The corresponding p-value for Ctrl and PD4 is 0,024, while p-value for PD1 and Ctrl is 0,002. When correcting for pixel intensity, the p-value between Ctrl and PD4 is 0,017 and the p-value between PD1 and Ctrl is 0,005. Movies are available as Movie S2-4.

Whole image analysis was validated by small frame analysis

While whole image analysis has the benefit of unbiased analysis, a downside of using automated trafficking software is the inability to distinguish between mitochondrial movement and (particularly sideways) movement of axons (see Supplemental figure S3D-E). It should be noted that these events take place in both patient and control cells, but we have chosen to further analyse data in smaller excerpts that showed no sideways movement during frame acquisition. To improve the accuracy of analysis, we have chosen to analyse excerpts away from the cell body, thereby focussing on axonal mitochondrial trafficking dynamics. Analysis of 20 smaller frame images, where no sideways movement occurred, again resulted in a significant difference in trafficking between PD and control lines (see supplemental figure S5). Since axonal excerpts with mitochondria are analysed, trafficking speed was higher than those observed in whole image analysis. These results confirm that mitochondrial trafficking in DA neurons of PD iPSC lines is impaired in comparison to mitochondrial trafficking in DA neurons of control iPSC lines. The corresponding p-value for Ctrl and PD4 is 0,001 and the p-value for PD1 and Ctrl is 0,001 in both percentage of pixels and percentage of pixel intensities moving.

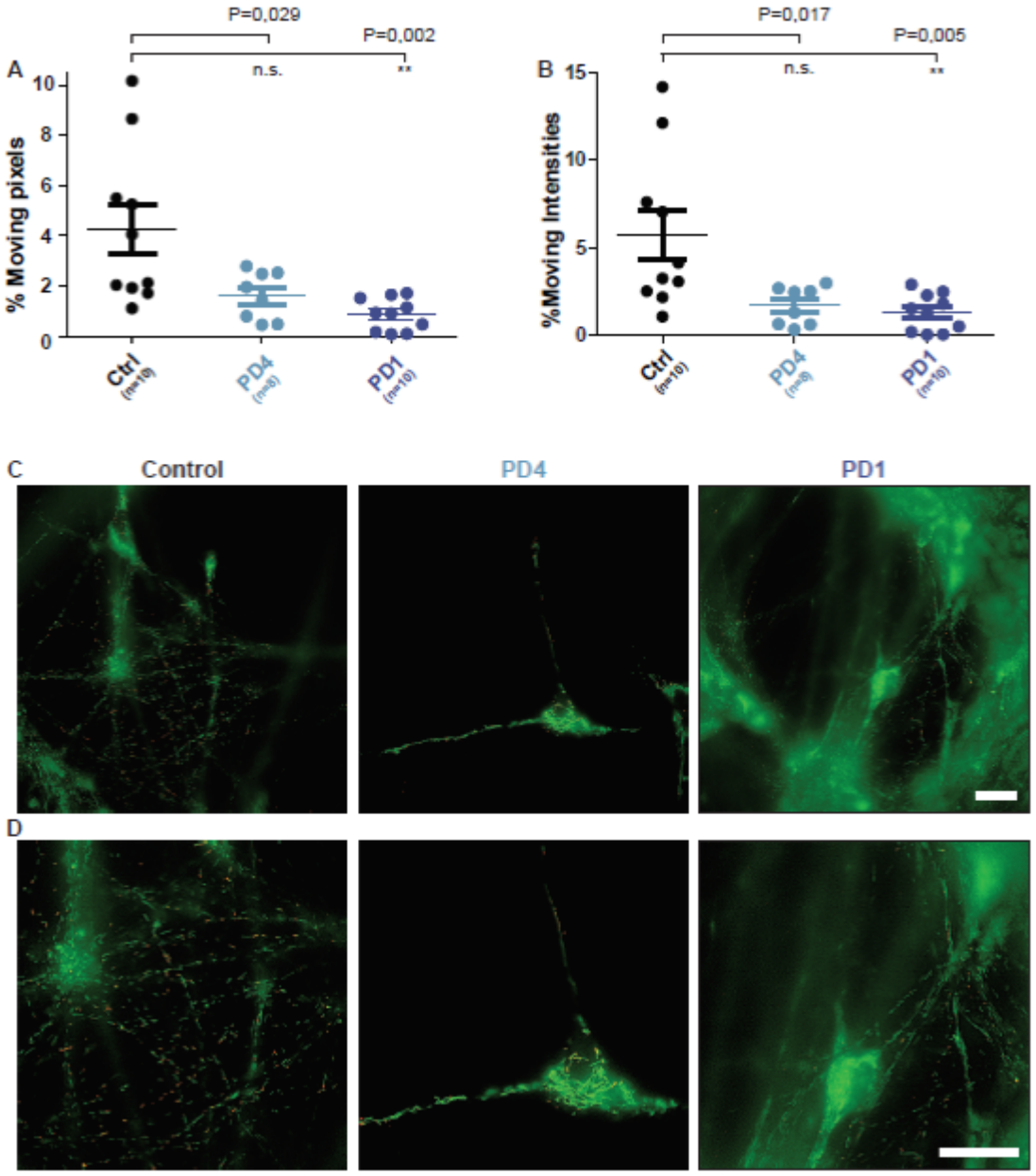

Figure 4: PD lines show mitochondrial trafficking impairment (A) Movement of mitochondria referenced to pixels shows a significant difference between control and PD lines (Ctrl vs. PD4 p=0,049 and Ctrl vs. PD p=0,006), but not between PD lines. (B) Moving intensity percentages are significantly different between control and PD lines (Ctrl vs. PD4 p=0,023 and Ctrl vs. PD p=0,007), but not between PD lines. (C) Example of mitochondrial trafficking analysis of control. (D) Excerpt of (C) shows less trafficking in PD4 and PD1 compared to control. Red indicates moving mitochondria, while green indicates stationary mitochondria. (Scalebar 25µm). Corresponding kymographs are illustrated in supplemental figure S4.

PD lines show mitochondrial fragmentation

In neurons, healthy mitochondria are typically elongated and undergo fusion and fission to maintain proper mitochondrial viability and functionality [15]. In our experiments, patient-derived PD DA neurons showed increased fragmentation of mitochondria (figure 5B-C), while control DA neurons (figure 5A) show healthy, elongated mitochondria. The increased presence of fragmented mitochondria hints to lowered mitochondrial integrity.

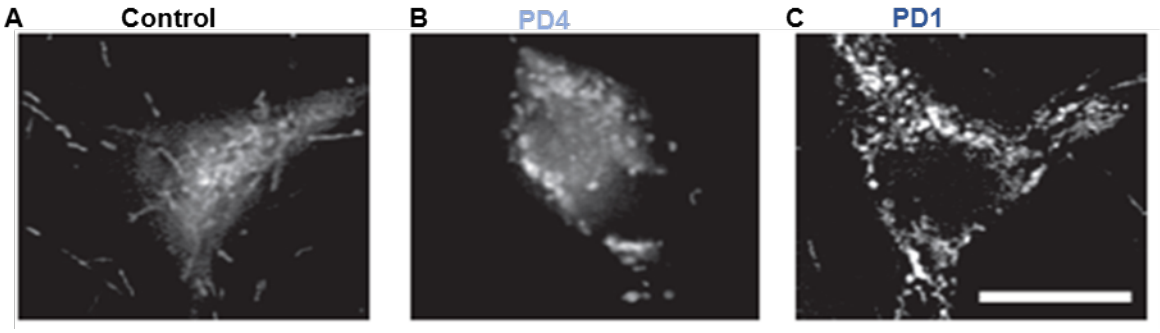

Figure 5: Mitochondrial fragmentation is observed in PD4 and PD1 line, but not in control (A) In all imaging conditions, control line mitochondria showed an elongated phenotype, while in the PD4 (B) and PD1 (C) line mitochondrial fragmentation and round mitochondria were observed (bars 10μm).

DISCUSSION AND CONCLUSION

In our experiments, we have demonstrated that mitochondrial trafficking is impaired in familial PD neurons generated from iPSCs compared to healthy age-matched control cells. Our data extends to previous data published by our group (Melo, van Zomeren et al. 2017) and supports the hypothesis that intracellular trafficking dynamics impairment precedes the onset of PD (Hunn, Cragg et al. 2015). Being able to culture pure neuronal cultures for extended periods of time has given us the opportunity to study mitochondrial trafficking without the use of environmental toxins, when compared to other PD studies (Reinhardt, Schmid et al. 2013, Ryan, Dolatabadi et al. 2013, Mazzulli, Zunke et al. 2016, Zagoura, Canovas-Jorda et al. 2017). Use of environmental toxins has led to a link between α-synuclein and mitochondrial pathology in previous studies, but these approaches mainly highlight the vulnerability of patient

DA neurons for these toxins. In contrast, our data gives insight to early pathology and mitochondrial dysfunction as opposed to a rapid onset of the disease phenotype. While mutations in parkin (PARK2) and PTEN-induced kinase 1 (PINK1) have provided a strong link between mitochondrial dysfunction and PD in iPSC studies (Chung, Kishinevsky et al. 2016), a link to early mitochondrial dysfunction in SNCA-mutated iPSCs has remained elusive.

Acknowledgements

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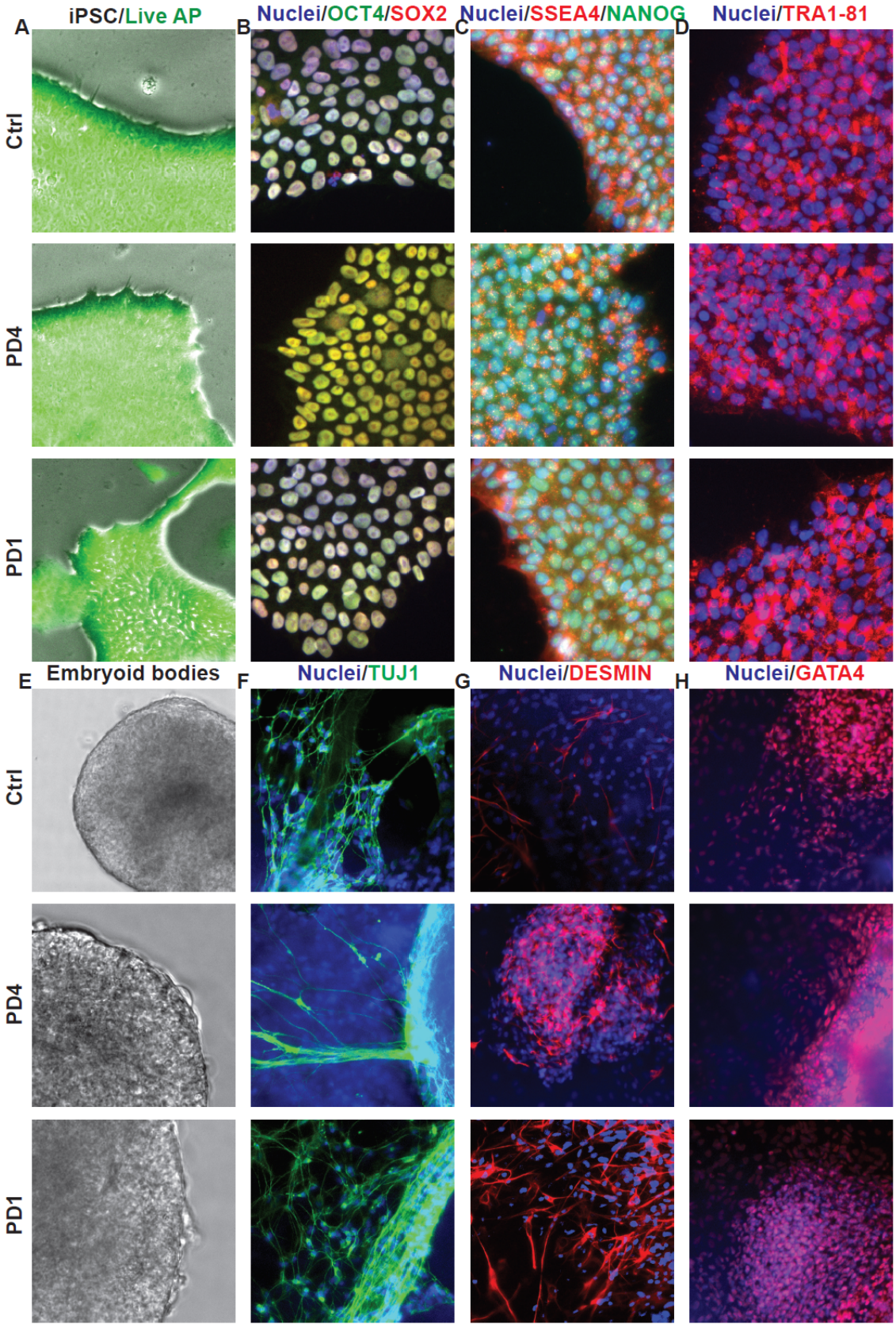
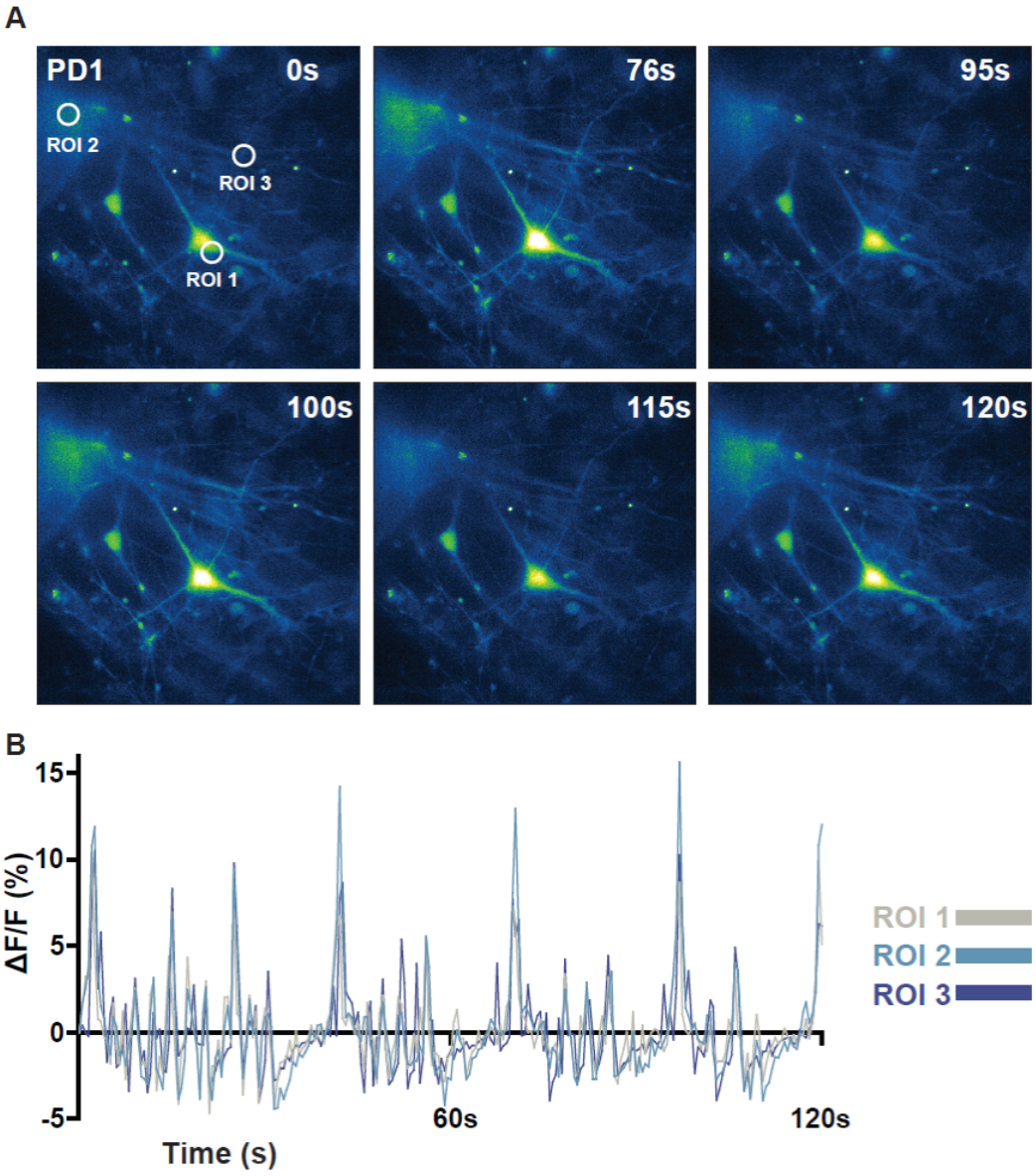
Supplementary information for chapter 5:



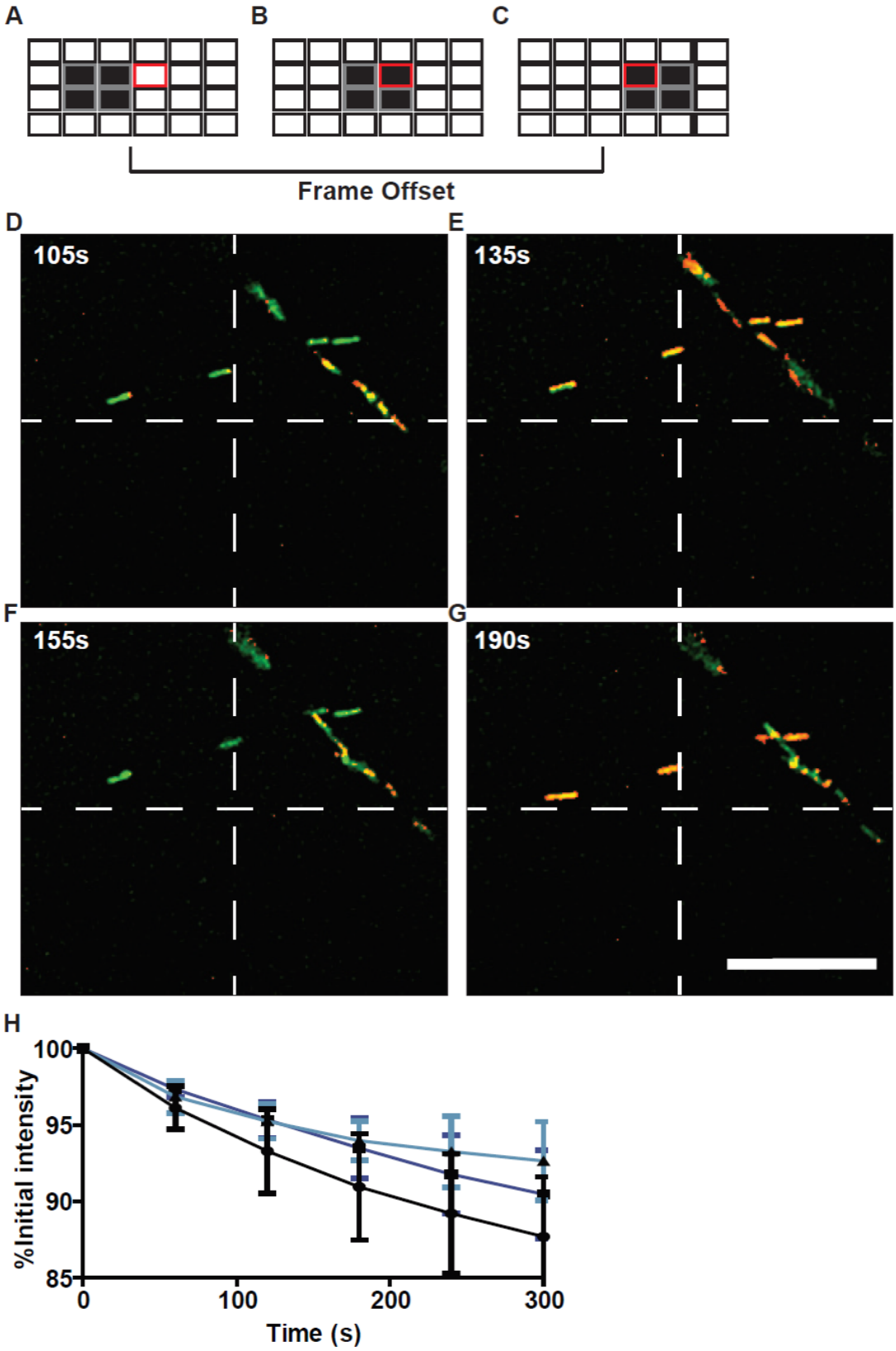
Supplemental figure S1. Characterization of iPSC lines.

(A) iPSC cultures stained with Live Alkaline Phosphatase staining (Thermo Scientific). **(B-D** iPSC immunocytochemistry for pluripotency markers. **(B)** Staining for OCT4

(green), SOX2 (red) and Hoechst (blue). **(C)** Staining for NANOG (green), SSEA4 (red) and Hoechst (blue) **(D)** Staining for TRA1-81 (red) and Hoechst (blue) **(E)** Embryoid body formation in all lines. **(F-H)** Embryoid body immunocytochemistry for DESMIN (red) and Hoechst (blue) **(F)**, TUJ1 (green) and Hoechst (blue) **(G)** and GATA4 (red) and Hoechst (blue) **(H)**.



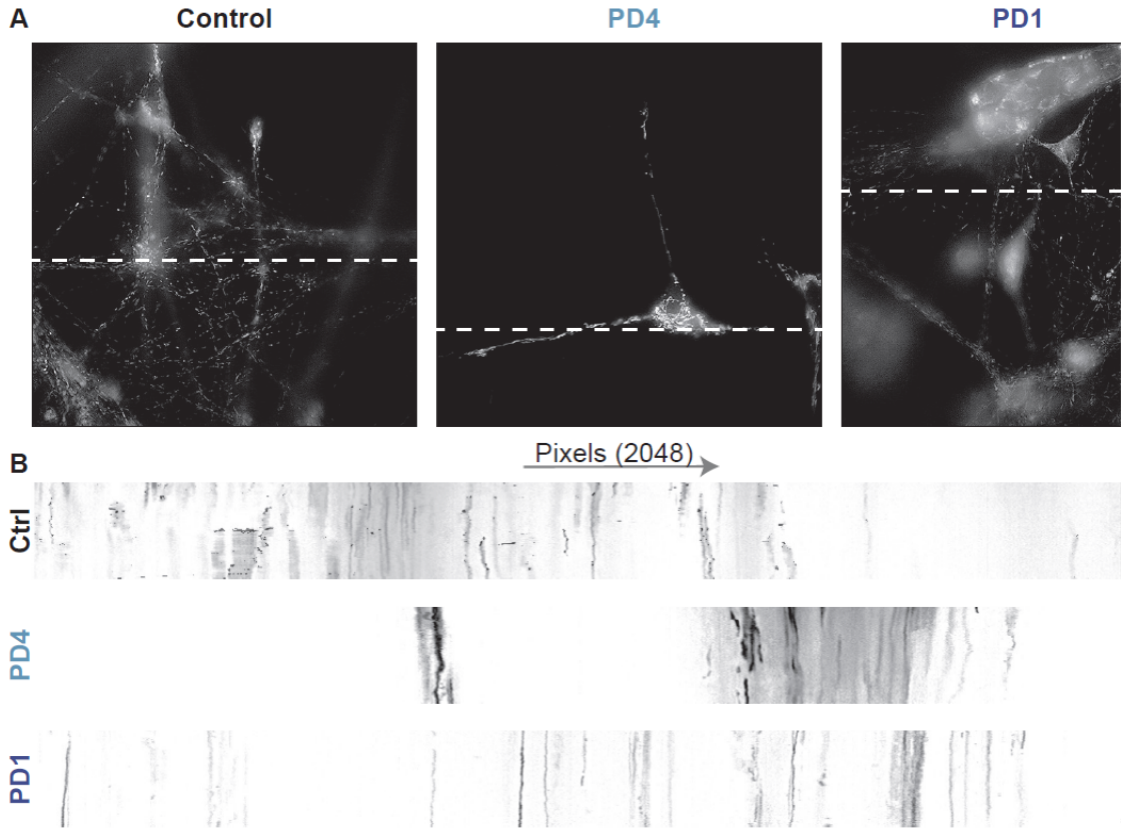
Supplemental figure S2. Calcium imaging of PD1 line at 90 DIV shows spontaneous neuronal activity patterns.
(A) Shows PD1 derived neuronal cells labeled with Fluo-4 AM over a period of 120 seconds. Spontaneous depolarization events are characterized by increasing presence of the green color. **(B)** Depicts the change in pixel values corresponding to ROI1, ROI2 and ROI3, showing similar activation patterns of these different locations. Data is represented as $\Delta F/F_0$.



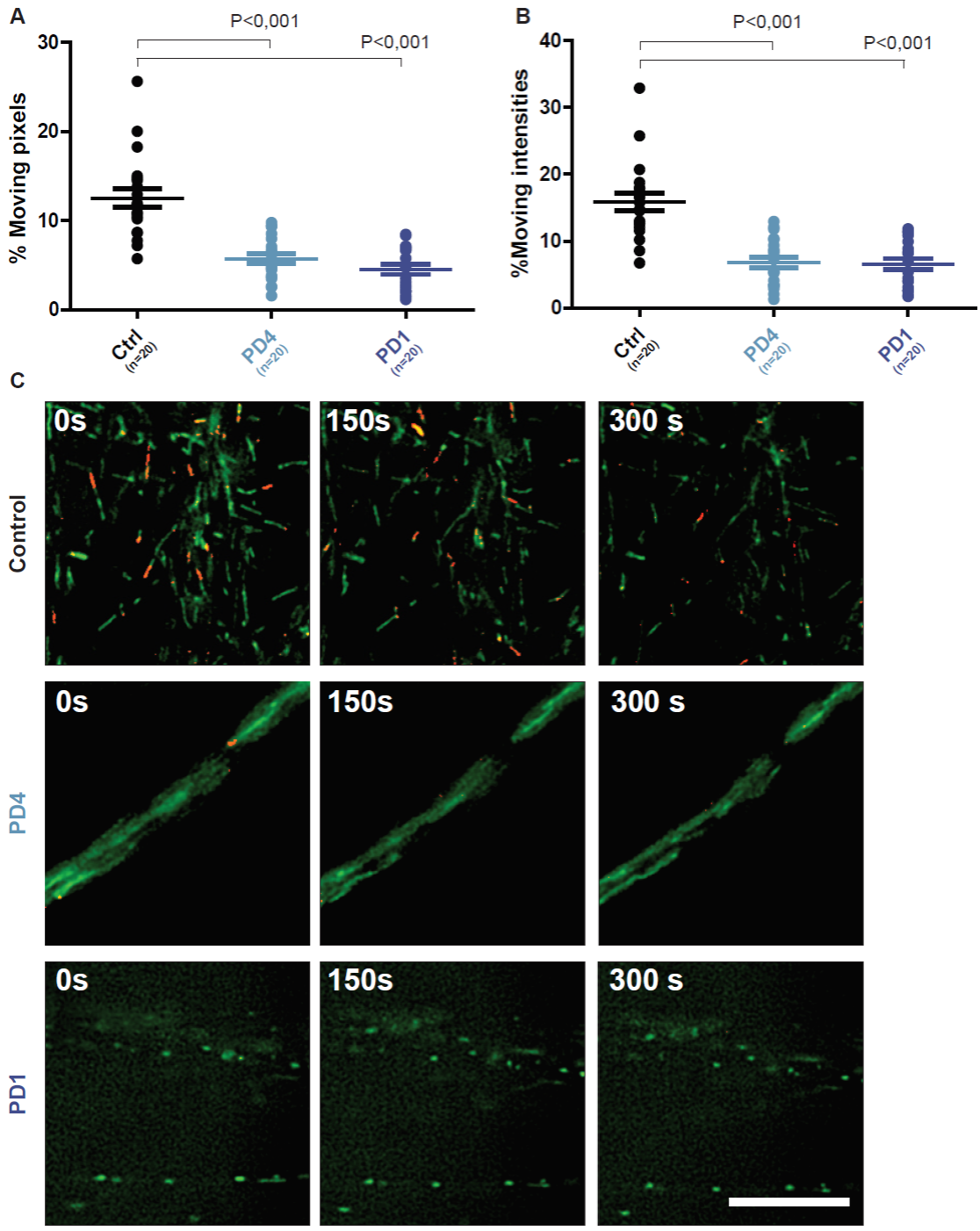
Supplemental figure S3. Simplified example of difference tracker software and experimental validations

From **(C)** to **(B)**, the plugin is unable to observe changes in the red boxed pixel, since it still records a positive pixel value in this frame. However, when looking at **(A)** a

difference is observed, and the pixel can be classified as moving. It should be noted that in the actual 8-bit image, as opposed to this binary example, the pixel can have 256 intensity values, allowing for offset values to classify pixels as moving. **(D)** An axonal shift results in a positive trafficking event **(E),** while in reality mitochondria are stationary. The axonal positions in **(F)** shift down **(G)** again resulting in false positive events. Since the software is not able to recognize these events, screening of smaller excerpt has to be performed to prevent a false positive bias of results. **(E)** Fluorescence intensity loss shows bleaching effects observed during imaging. While minor differences between groups were observed, the difference in bleaching was not significant, and in amounted on average 9,8%.



Supplemental figure S4. Kymographs of trafficking experiments
(A) Grey scale images containing MitoTracker® Orange labelled neuronal cells from control and PD patient lines. **(B)** Kymographs corresponding to figure 4, consisting of 117 frames, containing an average of a 5 pixel line indicated in the images depicted in **(A)**.



Supplemental figure S5. Trafficking impairment is observed when analyzing more detailed images

(A) Movement of mitochondria referenced to pixel intensities shows a significant difference between control and PD lines, but not between PD lines.

(B) Absolute moving percentages are significantly different between control and PD lines, but not between PD lines.

(C) Example of mitochondrial trafficking analysis of control. **(D)** Excerpt of **(C)** shows less trafficking in PD4 and PD1 compared to control. Red indicates moving mitochondria, while green indicates stationary mitochondria. (Scalebar 10µm).

CHAPTER 6

ABSENCE OF GEM (MIRO) REDUCES ALPHA-SYNUCLEIN TOXICITY IN A YEAST MODEL TO STUDY PARKINSON´S DISEASE

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Abstract

Alpha-synuclein is the main constituent of Lewy bodies, which are protein clusters characteristic of Parkinson´s disease (PD). Point mutations in the protein generating A30P or A53T alpha-synuclein are known to exacerbate the toxicity of alpha-synuclein causing more severe cellular damage. Mitochondrial dysfunction, aberrant autophagy, and endoplasmic reticulum (ER) stress can be caused by alpha-synuclein toxicity. Gem (the yeast orthologue of mammalian Miro) coordinates mitochondrial dynamics and cross-talk with the ER, which is impaired in the presence of mutant alpha-synuclein and can subsequently lead to disturbed autophagy and ER stress. *Saccharomyces cerevisiae* (yeast) is widely used to investigate conservative cellular mechanisms that are altered in neurodegenerative disorders such as PD. In this study, we expressed A30P or A53T alpha-synuclein in normal yeast or in yeast in which the *Gem* gene was knocked-out (ΔGem). We found that in control yeast cells, A53T alpha-synuclein forms more aggregates than A30P alpha-synuclein and increases the likeliness of cell death. Moreover, the expression of both mutant alpha-synuclein impaired normal yeast growth, led to increased mitochondrial H_2O_2 production, and increased levels of glutathione disulfide (GSSG) and ER stress. However, the absence of Gem prevented high consumption of glutathione (GSH), decreasing the GSH/GSSG ratio. In the presence of both mutant alpha-synucleins, the Δ*Gem* cells showed an increased growth and autophagic flux, preventing the formation of aggregates. The ratio of spliced and unspliced forms of *Hac1* decreased, indicating that the deletion of *Gem* could alleviate ER stress, despite the activated UPR machinery due to increased *Pdi1* and *Ero1* levels. Moreover, Δ*Gem* cells expressing A53T alpha-synuclein showed similar levels of mitochondrial H_2O_2 production as control cells, indicating that the absence of Gem prevented mitochondria dysfunction. Together, our results suggest that alpha-synuclein toxicity is dependent on *Gem* and that its deletion prevents cellular damage due to mitochondria dysfunction, inefficient autophagy, and ER stress, caused by the mutant alpha-synuclein.

INTRODUCTION

The general life expectancy is increasing and consequently aging-related diseases are becoming more common. Parkinson's disease (PD) is considered the most common age-related motor-neurodegenerative disease in the world (de Lau and Breteler, 2006) and is characterized by the presence of Lewy bodies and degeneration of dopaminergic neurons (DA) in the substantia nigra. Alpha-synuclein is the main component of Lewy bodies and it is thought to play a key role in the events linked to degeneration during PD (Giacomelli et al., 2017). Overexpression of alpha-synuclein or the expression of point-mutated forms, such as A30P or A53T alpha-synuclein, increase the propensity of oligomerization and aggregation of protein, which impairs mitochondria and endoplasmic reticulum (ER) function and leads to aberrant autophagy (Bose and Beal, 2016; Button et al., 2017; Mazzulli et al., 2016). It has been reported that A53T alpha-synuclein is more toxic and rapidly increases levels of reactive oxygen species (ROS) causing cell death (Bose and Beal, 2016; Colla et al., 2012; Smith et al., 2005). Impairment of mitochondrial dynamics has been found in the post-mortem brains of PD patients and has also been shown in several PD models (Das and Sharma, 2016). Mitochondrial dynamics are dependent on Gem (the yeast orthologue of mammalian Miro), a calcium-dependent motor/adaptor protein associated with the mitochondrial outer membrane and a target for all proteins related to mitochondrial fission, fusion, and autophagy among other events (Aresta et al., 2002; Chen et al., 2015; Devine et al., 2016; Wang et al., 2011). During autophagy, Atg8 (LC3 in mammals) triggers the formation of autophagosomes and degraded after fusion with a degradation vacuole in yeasts. Defectives in the mitochondria autophagy could impair the cellular homeostasis by impair ER function. Gem is also localized in contact sites between the mitochondria and ER and mediates the cross talk between these organelles (Bockler and Westermann, 2014; Devine et al., 2016; Friedman et al., 2010; Hamasaki et al., 2013; Xie and Chung, 2012). It has been shown that A30P and A53T alpha-synuclein diminish the contact sites between the organelles leading to the accumulation of defective mitochondria by impairing autophagy. Defective mitochondria produced high levels of ROS and through cross-talking with the ER lead to the organelle stress, which also start releasing high levels of ROS (Cali et al., 2012; Guardia-Laguarta et al., 2014; Manor et al., 2015).

Glutathione (GSH) is the main molecule responsible for maintaining the redox state in ER, mitochondria, and cytoplasm. When wild-type (WT), A30P, or A53T alpha-synuclein accumulates in the ER, or mitochondria start releasing high levels of ROS, GSH oxidizes and activates the unfold protein response (UPR) to restore ER homeostasis. In yeast, Ire1 triggers the UPR that activates the chaperone disulfide isomerase (Pdi), the main protein involved in the folding protein machinery of the ER. Pdi overexpression is one of the main markers of ER stress. During the protein folding process, Pdi oxidizes and reduces using the enzyme endoplasmic reticulum oxidoreductase 1 (Ero1) generating hydrogen peroxide (H_2O_2). Additionally, accumulated protein in the ER favors calcium leakage to cytosol (Feissner et al., 2009; Haynes et al., 2004; Malhotra and Kaufman, 2007; Tu and Weissman, 2004). Mitochondria uptakes the excessive calcium released from the ER and consequently, its metabolism increases as well as the production of H_2O_2. In DA neurons, the UPR also activates the x-box binding protein 1 (XBP1), yeast ortholog Hac1, which plays a role in activating gene expression to promote neuron survival. Alternatively, in the long-term presence of excessive or misfolded proteins, Ire1 gain a RNase activity promoting the alternative splicing of Hac1 mRNA, which is another main marker of ER stress (Delic et al., 2012; Grimm, 2012; Krols et al., 2016; Mercado et al., 2016; Nikawa et al., 1996; Szegezdi et al., 2006; Zeeshan et al., 2016).

Alpha-synuclein toxicity has been widely investigated, however, the role of Gem and the link among mitochondrial, ER and autophagy dysfunction in the presence of alpha-synuclein is still unclear. In this study, we used *Saccharomyces cerevisiae* as a yeast humanized model Δ*Gem* (no expression of *Gem*) to investigate the role of Gem in A30P and A53T alpha-synuclein toxicity by mitochondrial and ER stress.

MATERIAL AND METHODS

Transformation and viability and sensitivity essay of *Saccharomyces cerevisiae*

The *Saccharomyces cerevisiae* strain used in this study was BY4741 (genotype: Matα; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YHR104w: kanMX4). The Δ strain used was Δ*Gem1* (homologous to human Miro) derived from BY4741. Cell lines were obtained

from the Euroscarf collection (Frankfurt, Germany), kindly donated by professor Luis Eduardo Soares Netto from the Institute of Biosciences-University of São Paulo. The strains were transformed using p426GPD empty plasmid (empty vector, cited as E.V.), or containing A30P or A53T alpha-synuclein (cited as A30P or A53T), and they were donated by Professor Susan Lindquist (Outeiro and Lindquist, 2003). Both genes are under control of the constitutive promoter GPD, Glyceraldehyde-3-phosphate dehydrogenase. It was obtained the followed groups: BY4741 or Δ*Gem* expressing empty vector, cited as B. E.V. or G.E.V., or expressing A30P alpha-synuclein, cited as B. A30P or G. A30P, or expressing A53T alpha-synuclein, cited as B. A53T or G.53T, respectively. Selection markers of plasmids were ampicillin and uracil for bacteria and yeast, respectively. Yeast cells grew overnight on YPD containing 1% yeast extract, 2% peptone, 2% glucose or Synthetic Dropout Medium (SD) containing 0.67% yeast nitrogen base without amino acids, 2% glucose or galactose as carbon source and 0.13% amino acid mix. Cells were transformed with plasmids using the lithium acetate method based on Schiestl and Gietz (Schiestl and Gietz, 1989).

Cells viability was analyzed through growth curves and serial dilution in fermentation medium, which is synthetic defined minimal medium (SD) containing 20% of glucose, 1.7% of nitrogenous base, 1.3% of dropout which consisted of 0.67% yeast nitrogen base without amino acids, 2% glucose (SD), galactose (SG) or glycerol and ethanol (SGE) as carbon source and 0.13% amino acid mix) in the absence and presence of H_2O_2. Strains grew overnight in SD for 24h at 34°C in shaker, then diluted at 0.2 OD (optical density) and OD_{600} was measured at 0h, 2h, 4h, 6h, 8h, 10h, 12h and 24h. In serial dilution, strains were plated from 1.0 OD_{600} in tenfold serial dilutions in solid SD medium for 4 days. Sensitivity assay was performed with control line BY4741. Cells were plated onto dishes containing 0mM, 0.5mM, 1.0mM, 1.5mM, 2mM, or 3mM of H_2O_2. After 8 days, yeast growth was observed and registered through photomicrographs.

Subcellular fractionation and alpha-synuclein localization

In analyses when mitochondrial activity was necessary, we used SD medium in which glucose was replaced by galactose (SG medium) allowing the respiration and fermentation processes. Cells grew in SG medium and mitochondria, ER, nuclei and

cytosol were fractionated as previously described by Rieder and Emr (Rieder and Emr, 2001). Alpha-synuclein localization was observed in the 6 fractions: total extract of cells (EX), no lysed cells, P_{1000} where oligomers, aggregates and nuclei can be found, isolated mitochondria extract (Mit) and isolated ER extract (ER). Western blotting was performed to assure that the protocol was effective and to identify in which fraction alpha-synuclein was present.

Western blotting

Three OD_{600} of cells were centrifuged at 1600 × *g* for 1 min at 4 °C. Cells were resuspended in ultrapure water followed by the addition of 2 M NaOH (sodium hydroxide) and 7.4% β-mercaptoethanol. Subsequently, cells were incubated on ice for 10 min, then mixed with 50% trichloroacetic acid (TCA), followed by 10 min of incubation on ice. Cells were centrifuged at 16000 × *g* for 2 min at 4 °C, and the pellet was washed with 500 µl of 1 M Tris-Base. Samples were centrifuged at 16000 × *g* for 10 seconds at 4 °C. The pellet was resuspended in 5× Laemmli sample buffer (60 mM of Tris-HCl pH 6.8, 20% glycerol, 2% SDS, 14.4 mM β-mercaptoethanol, and 0.1% bromophenol blue). DTT (1 mM) was added, and samples were incubated at 95 °C for 5 min followed by incubation on ice. Thirty µg/lane of each sample was fractioned in 12% Tris-HCl SDS-PAGE gels. Next, proteins were transferred to nitrocellulose. Membranes were blocked with 5% milk (Blotting Grade Blocker Non-Fat Dry Milk, BioRad) in TBS-T for 1 h at room temperature. To verify alpha-synuclein expression or localization and specific cellular fractions, membranes were incubated overnight at in TBS-T with the anti-alpha-synuclein (sc-7011R, Santa Cruz Biotechnology; 18 kDa) at 1/1000, Pgk1 antibody as a cytoplasm marker (ab113687, Abcam; 44 kDa) incubated at 1/10000, Dmp1 as an ER marker (MABD10, Life Technologies; 65kDa) incubated at 1/2000 or with anti- porin1 as a mitochondria marker (16G9E6BC4, Life Technologies; 35 kDa) at 1/1000. HRP-conjugated secondary antibodies used were anti-mouse (Amersham) at 1/6000 or anti-rabbit (1/10000) for 1h at room temperature in TBS-T. ECL™ Prime Western Blotting Detection (GE Healthcare) was used to visualize antigen-antibody complexes, followed by exposure to appropriated films (Hyperfilm ECL, Amersham Biosciences). ImageJ software (National Institutes of Health, USA) was used to quantify film data.

H_2O_2 levels measurement through Amplex Red™ oxidation in isolated mitochondria

Mitochondrial isolation followed the protocol of Glick & Pon (1995). Briefly, samples with 100 μg/ml of suspension containing mitochondria were incubated with 50 μM of Amplex Red (Molecular Probes) in the presence of 1.0 U/ml of horseradish peroxidase (HRP, Sigma-Aldrich) for 10 min in a shaker at 30 °C inside a fluorimeter (Cary 100 Bio, Varian). The quantitative values of H_2O_2 in arbitrary units of fluorescence were calculated from a calibration curve with different concentrations of H_2O_2 diluted 1/1000 from 30% of the chemical (v/v).

HPLC (High Performance Liquid Chromatography) with electrochemical detection

The ratio between glutathione in reduced form (GSH) and oxidized form (GSSG) were measure using cells grown overnight in SD and then for 24h in SD which glucose was replaced by glycerol and ethanol (SGE medium), allowing only respiration. Next, samples were centrifuged at 1000 × *g* for 5 min and cells were resuspended in sulfosalicylic acid at the same volume of the pellet. To lyse cells, glass beads were added to samples that were vortexed for 20 min at 4 °C. Extracts of samples were centrifuged at 16000 × *g* for 40 min, and the supernatant, was collected for analysis. To measure the GSH/GSSG ratio, the Coulochem III HPLC-ECD system (ESA, Inc.) equipped with one guard cell (model 5020) and analytic cell with electrode BDD (boron doped diamond, model 5040) was used. Component elution was monitored by applying a potential of +1400 mV in the analytic cell and +900 mV in the guard cell, finalizing loading by applying a potential of +1900 mV for 30 s followed by rebalancing for 5 min. GSH was used at 0, 0.5, 1, 2.5, 5, 7.5, and 10 mM and GSSG at 5, 10, 25, 50 mM, 75, and 100 mM as quantitative standards to construct a calibration curve. Supernatants were filtered and 50 μl of each sample was injected in the machine. Chromatograms of total thiols and disulfide detections were analyzed, and the GSH and GSSG peaks were identified and quantified.

Autophagy monitoring by GFP-Atg8 fusion analysis

Strains were transformed with the centromeric plasmid pCuGFPATG8415 which expressed GFP-Atg8, using the lithium acetate method based on Schiestl and

Gietz (Schiestl and Gietz, 1989) as describe above. The levels of GFP-Atg8 and GFP free were analyzed by western blotting as described above, using the anti-GFP (Sigma) antibody incubated at 1/20000 overnight in TBS-T.

Evaluation of UPR (unfolded protein response) and endoplasmic reticulum (ER) stress through alternative splicing of *Hac1* and expression of Pdi and Ero1

Reverse transcription PCR

To evaluate unspliced and spliced *Hac1* levels, strains grew in SGE medium and total RNA was extracted using hot phenols following a protocol published by Scherrer & Darnell (1962). *Hac1* levels were evaluated through cDNA amplification using 2 µl of cDNA at 1 µg/µl, 1 µl of 10 µM forward primer (TACAGGGATTTCCAGAGCACG), 1 µl of 10 µM reverse primer (TGAAGTGATGAAGAAATCATTCAATTC), 1.5 µl of 10 mM dNTP mix, 5 µl of 10× PCR buffer, 1.5 µl of $MgCl_2$ at 50 mM, 0.5 µl of Taq DNA polymerase at 5 U/L, and ultrapure water to dilute the solution to 50 µl. Samples were denatured at 94 °C for 2 min, followed by 22 denaturation cycles at 94 °C for 30 seconds, an annealing cycle at 54 °C for 30 seconds, and an extension cycle of 72 °C for 10 min. Samples were loaded into a 1% agarose gel, and images were analyzed in ImageJ (National Institutes of Health, Bethesda, Maryland, USA). cDNA of ADH4 (1369 base pairs) was amplified using the proper forward primer (TCACGACAATGCTAAGGCA) and reverse primer (AACACCATGAGGCAAGTGGT) and was used as a control to normalize *Hac1* expression, followed by the calculation of the ratio between the spliced form of ^{S}Hac1 (processed form of 450 base pairs) and the unspliced form of ^{U}Hac1 (large form of 651 base pairs).

qRT-PCR

Quantitative real-time PCR was performed to analyze the expression of Pdi and Ero1 genes and the housekeeping gene Act1. Strains grew in SD medium and cDNA was obtained as described above and was amplified using the following forward primers: AGTTATCGTCCAATCCGGTAAG, AACGCCGTTCTGATTGATTT and TTCCCAGGTATTGCCGAAA and reverse primers: GCGGAGGGCAAGTAAATAGA, GATTCACCAGTTTCGCCAAT and TTGTGGTGAACGATAGATGGA for Pdi, Ero1 and Act1, respectively. The reactions were performed using SYBR Green Master Mix

(Applied Biosystems) mixed with forward and reverse primers and cDNA following manufactures intructions. The Applied Biosystems 7500 was used to detect the curves amplification that were analyzed in software 7500 System SDS v.1.2 (Applied Biosystems). The ΔCt, ΔΔCt and the final relative expression $2^{-\Delta\Delta Ct}$ were calculate and statistic analyzed.

Statistical analyses

Results were analyzed by one-way ANOVA followed by Tukey's post-hoc test or *t* Test using GraphPad Prism (GraphPad Software Inc., version 4.00, CA). Differences were considered statistically significant at a p-value of ≤ 0.05. All data are expressed as percent of control or absolute values ± standard deviation (SD).

RESULTS

Transformation and viability of yeast strains

To create a humanized yeast model to study PD, cells strains were successfully transformed with A30P or A53T alpha-synuclein. In order to verify alpha-synuclein expression in the cells, a protein extraction protocol was used based on NaOH which promotes increased permeabilization of the cell wall and total protein breakdown; this procedure results in an extraction ready to be loaded unto an SDS-PAGE gel. It is not an extraction method that can be used to analyze protein aggregates as it potentially causes the breakdown of aggregates (Zhang et al., 2011). As expected, all strains showed an 18kDa band for both mutant alpha-synuclein proteins, corresponding to the monomeric form of alpha-synuclein. Interestingly, BY4741 expressing A53T alpha-synuclein showed an additional band in the stacking gel for A53T alpha- synuclein, indicating protein aggregation. The A53T band was absent in the stacking gel of Δ*Gem* (Figure 1A). From now the control BY4741 strain and the deletion of *Gem* will be represented in the graphs as B. and G., respectively. The viability of cells was analyzed using a dilution series and a growth curve in three different conditions: 1) using a fermentation medium, SD; 2) using SG medium to allow yeasts to ferment and respirate; and 3) using SGE medium to allow yeasts to respirate only to obtain energy. In the SD medium, cells

grew for 4 days. Alpha-synuclein expression inhibited the growth of the BY4741 (Figure 1B). The deletion of the *Gem* gene inhibited yeast growth compared to control BY4741 expressing E.V. (control). Intriguingly, the expression of both A30P and A53T alpha-synuclein ameliorated Δ*Gem* cells growth (Figure 1C).

In the growth curve assay, A30P and A53T alpha-synuclein impaired the growth of BY4741 (Figure 1D). Following 24 h of growth, OD_{600} was measured again, which revealed that growth of BY4741 cells significantly decreased (Figure 1D1). *Gem* deletion significantly impaired normal growth of cells relative to BY4741 after 24 h (Figure 1H). However, the expression of A30P alpha-synuclein ameliorates the strain growth, while the expression of A53T alpha-synuclein did not change cells growth when compared with Δ*Gem* cells expressing E.V. (Figure 1E and 1E1). These results suggest that deletion of the *Gem* protect cells against impairment in the growth caused by the expression of mutant alpha-synuclein. However, when we compared Δ*Gem* cells grown in the solid medium for 4 days with those grown in the liquid medium for 24h it was clear that the expression of A53T alpha-synuclein delays yeast growth, which needed more than 24h to recover a normal growth range. In the viability assays using the SG medium, cells grew for 4 days. All cell strains grew less than cells grown in the SD medium, showing the yeast´s preference to grow in a fermentative environment (supplemental Figure 1A and B). In the dilution series performed with the SG medium, deletions in *Gem* gene impaired the normal growth of cells relative to the BY4741 cells (supplemental Figure 1B). Interestingly, A30P and A53T alpha-synuclein did not change Δ*Gem* growth (supplemental Figure 1B), while A53T alpha- synuclein inhibited BY4741 cells growth (supplemental Figure 1A). In the growth curve assay, deletion of *Gem* significantly inhibited the normal growth of cells relative to the BY4741 cells following 24h of growth (supplemental Figure 1E). A30P and A53T alpha-synuclein did not change BY4741 growth (supplementary Figure 1C and C1), whereas they both significantly ameliorated the growth of Δ*Gem* cells (supplemental Figure 1D and D1).

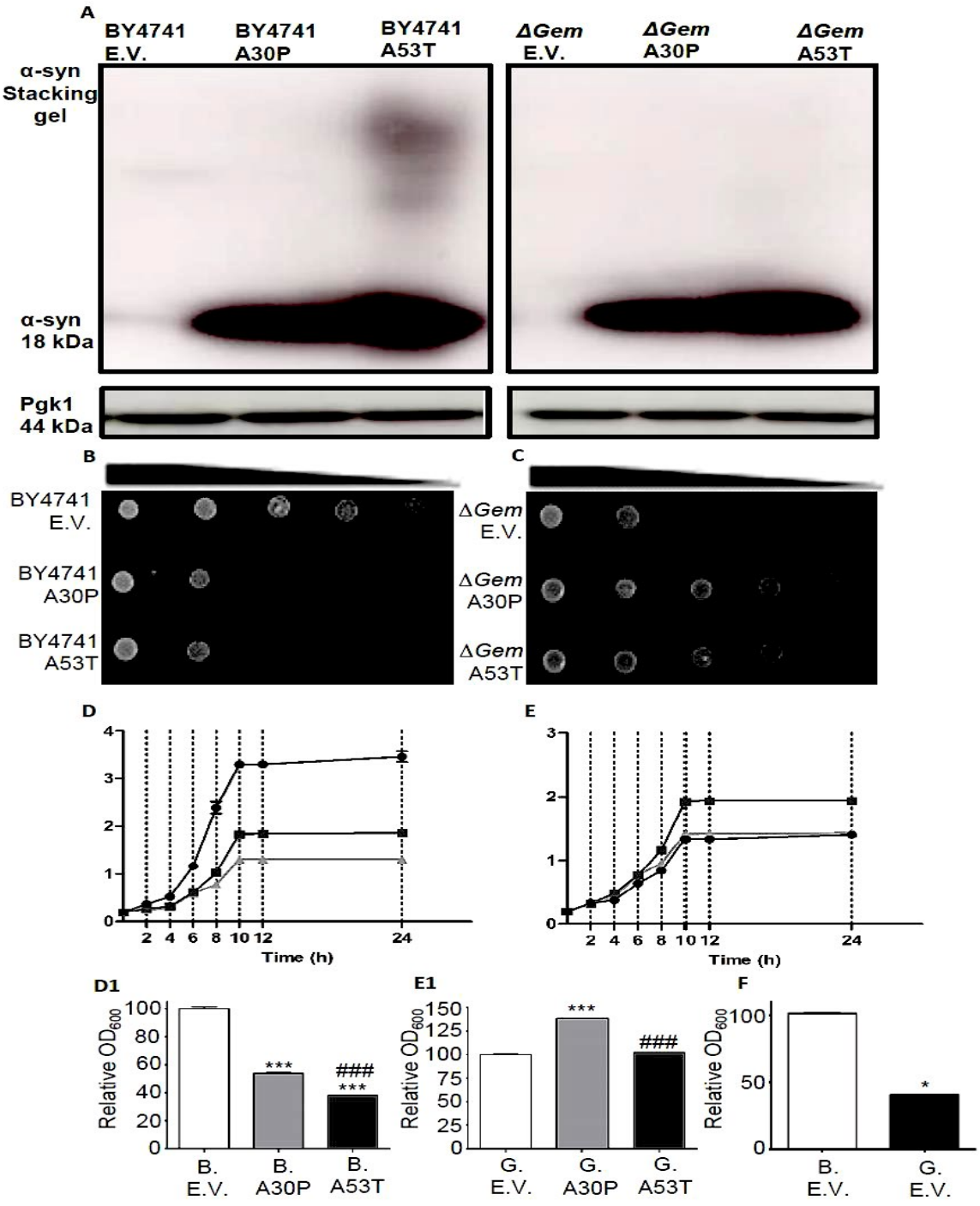

Figure 1. Alpha-synuclein (18kDa) and normalizer Pgk1(44kDa) expression and viability of cells expressing A30P or A53T α-synuclein. (**A**) Representative blotting of A30P and A53T α-synuclein expression from BY4741 and *ΔGem* cells. As expected, both strains showed an 18kDa band indicating expression. of monomeric alpha-synuclein. BY4741 cells expressing A53T alpha-synuclein showed another band, in stacking gel, indicating protein aggregation. (**B**) dilution series from a concentration at 1.0 OD, diluted at 1/10; 1/100; 1/1000; 1/10000 cultured for 4 days of BY4741. Cells

expressing mutant alpha-synuclein showed inhibited growth than cells expressing E.V. (**C**) dilution series from a concentration at 1.0 OD, diluted at 1/10; 1/100; 1/1000; 1/10000 cultured for 4 days of *ΔGem*. Cells expressing mutant alpha-synuclein showed ameliorated growth than cells expressing E.V. (**D and E**) growth curve from a concentration at 0.2 OD (shown on vertical axis) measured every 2h of BY4741 and *ΔGem* cells, respectively. (**D1**) Quantification of growth curve at 24h of BY4741. Cells expressing mutant alpha-synuclein showed inhibited growth than cells expressing E.V. (**E1**) Quantification of growth curve at 24h of *ΔGem*. Cells expressing mutant A30P alpha-synuclein showed ameliorated growth than cells expressing E.V. or A53T alpha-synuclein. (**F**) Quantification of growth curve at 24h comparing BY4741 and *ΔGem* cells expressing E.V. Absence of *Gem* impaired normal growth compared to BY4741. The values of 3 independent experiments (n=3) are expressed as percent to control (E.V.) ± SD. One-way ANOVA followed by Tukey post test (comparison of cells expressing E.V. or alpha-synuclein) or *t* test (comparison between BY4741 and *ΔGem*) were statistical test employed. *p≤0.05 compared with respective control. # p≤ 0.05 compared with cells expressing A30P alpha-synuclein.

In the SGE medium, all cell strains grew in a solid medium for 9 days. The SGE medium inhibited the growth of all cells (supplemental Figure 2). The growth of the BY4741 cells expressing A53T alpha-synuclein was completely inhibited (supplemental Figure 2A). However, A30P and A53T alpha-synuclein expression did not change Δ*Gem* growth (supplemental Figure 2B). It was not possible to perform growth curve assays on these samples because the cells did not grow in the liquid SGE medium. Even after 12 h of culture in liquid SGE medium, the OD_{600} did not change from 0.2.

Next, BY4741 cells were exposed to H_2O_2 for 10 days. Cells were cultured in an SD medium and a dilution series was performed to analyze the viability/sensitivity of the cells to H_2O_2. Δ*Gem* did not grow when exposed to H_2O_2 at 0.5mM or smaller concentrations. A sensitivity assay showed that in the absence of H_2O_2 or in the exposure of H_2O_2 at 0.5mM, A30P and A53T alpha-synuclein decreased BY4741 viability (supplemental Figure 3A and B). However, 1mM of H_2O_2 decreased the viability of all groups (supplemental Figure 3C). Furthermore, BY4741 cells expressing A53T alpha-synuclein, which were exposed to 1.5mM, 2mM or 3mM H_2O_2 showed lower viability than cells expressing A30P alpha-synuclein (supplemental Figure 3D, E and F). These data suggest that A53T alpha-synuclein is more toxic to cells than A30P alpha-synuclein.

Localization of oligomers and aggregates of alpha-synuclein in cellular fractionation

A30P and A53T alpha-synuclein mutations produce high levels of oligomers in cell lines (Marmolino et al., 2016). A53T alpha-synuclein oligomers interact with the membranes of organelles such as mitochondria (membrane-bound oligomers) (Miklya et al., 2014). In contrast, A30P alpha-synuclein does not have high membrane affinity (Ghio et al., 2016; Nakamura et al., 2008). It is possible to analyze the cellular fractions in which proteins clusters, oligomers and aggregates are present, therefore, we analyzed oligomers and aggregates presence and their interaction with organelles and membranes. In the BY4741 (control) and Δ*Gem* cells both mutant A30P and A53T alpha-synuclein were found in the subcellular fractions corresponding to total extract (EX) of no lysed cells or P_{1000} where oligomers, aggregates, and nuclei can be found, indicating both alpha-synuclein are oligomerized and aggregated. A53T and A30P alpha-synuclein were also found in the isolated mitochondria extract (Mit), indicating a possible interaction of the proteins with the mitochondrial membrane that could lead to mitochondrial dysfunction. Curiously, both mutant proteins from the control and Δ*Gem* were not found in the isolated ER fraction (Figure 2A and B).

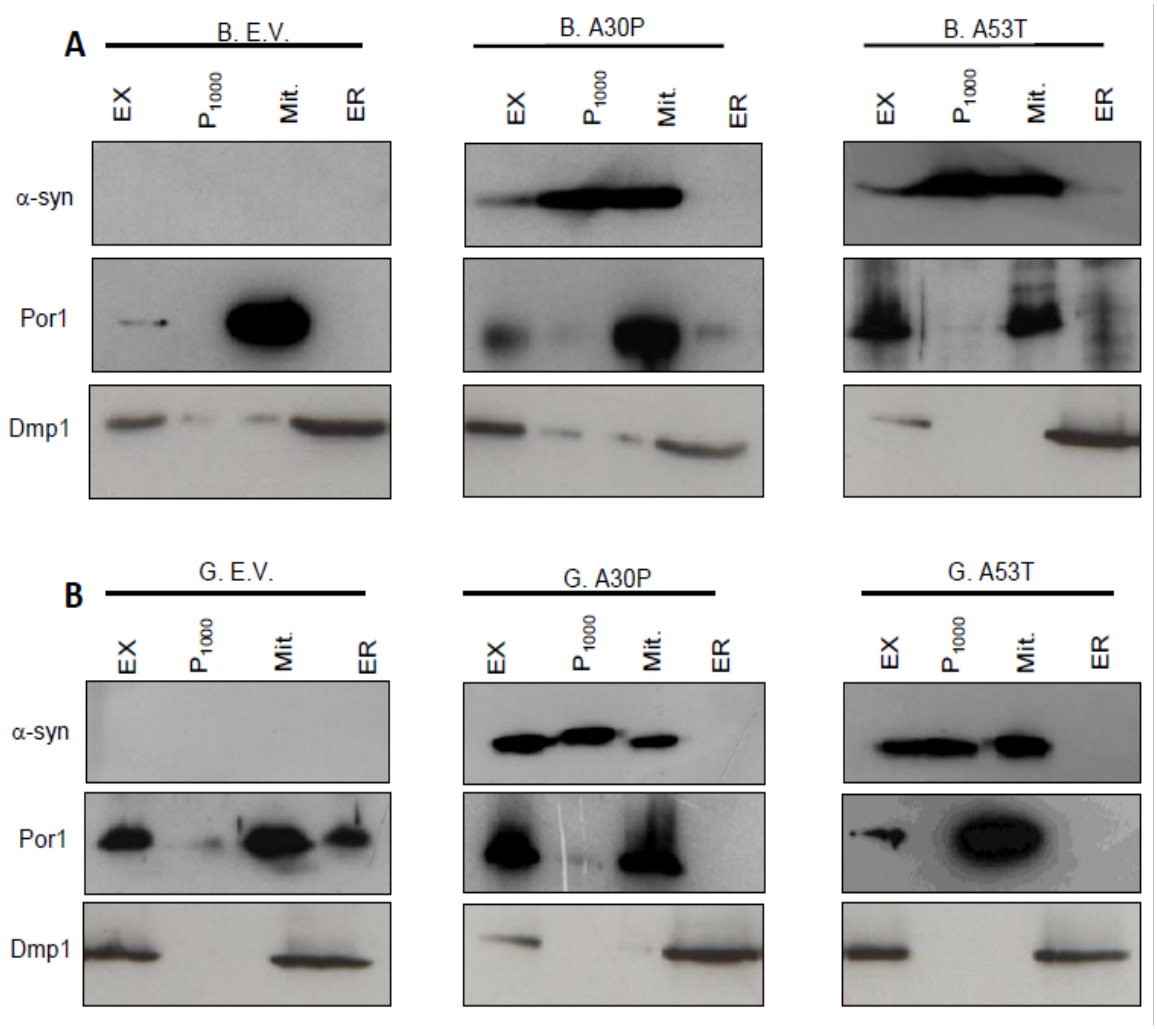

Figure 2. Representative blottings of cellular fraction of BY4741 and *ΔGem* cells and localization of alpha-synuclein (18kDa) in the fractions. Porine (30kDa) and Dmp1 (30kDa) were used to identify mitochondria and endoplasmic reticulum fractions, respectively. **(A)** Cellular fractions of BY4741 cells expressing E.V. or mutant alpha-synuclein. **(B)** Cellular fractions of *ΔGem* cells expressing E.V. or mutant alpha-synuclein. Alpha-synuclein was found in the fraction of total extract (EX), in the fraction containing no lysed cells, aggregates and nuclei (P1000) and in the fraction containing isolated mitochondria (Mit). A30P or A53T alpha-synuclein were not found in ER fraction.

H_2O_2 production of isolated mitochondria

The interaction of A53T oligomers with the mitochondrial membrane can lead to mitochondrial dysfunction and oxidative stress due to the high mitochondrial production of H_2O_2 (Chemerovski-Glikman et al., 2016; Byers et al., 2011). To investigate whether the presence of mutant alpha-synuclein could interfere with mitochondrial function,

mitochondria were isolated and the levels of H_2O_2 produced by mitochondria were analyzed. We found that, mitochondria from cells with the deletion of *Gem* gene produced higher levels of H_2O_2 in comparison to the mitochondria from BY4741 cells (Figure 3E). Mitochondria from BY4741 cells expressing A53T alpha-synuclein showed higher production of H_2O_2 than BY4741 cells expressing E.V. (Figure 3B) or A30P alpha-synuclein (Figure 3A). Conversely, mitochondria from Δ*Gem* cells expressing A53T alpha-synuclein produced lower levels of H_2O_2 than cells expressing E.V. (Figure 3D) or A30P alpha-synuclein (Figure 3C), suggesting that the absence of *Gem* was protective against the mitochondrial dysfunction caused by A53T alpha-synuclein.

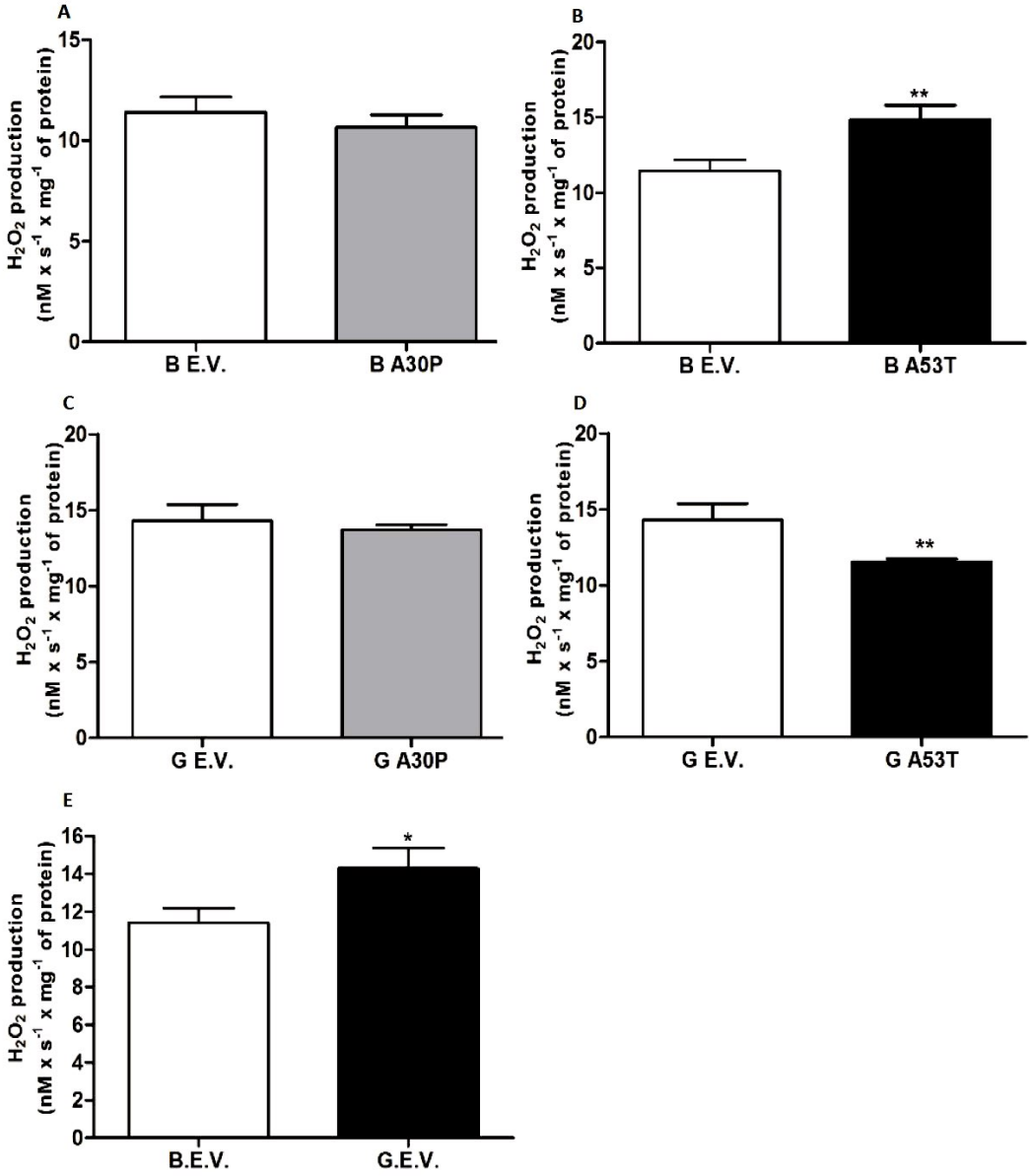

Figure 3. Quantification of H_2O_2 levels produced by mitochondria using Amplex Red. (**A**) A30P alpha-synuclein expression did not change the mitochondria H_2O_2 production of BY4741 cells. (**B**) Mitochondria from BY4741 expressing A53T α-synuclein, produced higher levels of H_2O_2 compared to cells expressing E.V. (**C**) A30P alpha-synuclein expression did not change the mitochondria H_2O_2 production of Δ*Gem* cells. (**D**) Mitochondria from Δ*Gem* cells expressing A53T α-synuclein produced lower levels of H_2O_2 compared to cells expressing E.V. (**E**) Δ*Gem* expressing E.V. showed higher levels of H_2O_2 compared to BY4741 expressing E.V. The values of 3 independent experiments (n=3) are expressed as absolute number ± SD and *t* test was statistical test employed. *p≤ 0.05 compared with respective control.

Total GSH/GSSG ratio measurements

Increased levels of H_2O_2 changes the redox state of cells and potentially leads to an imbalance between the reduced (GSH) and oxidized forms of glutathione (GSSG). To elucidate the redox state of the cells, the amount of GSH, GSSG, and the balance between the two glutathione forms, the GSH/GSSG ratio, were analyzed. The GSH/ GSSG ratio was significantly lower in the Δ*Gem* cells than in the BY4741 cells, indicating the deletion of *Gem* lead to higher levels of GSSG in the cells (Figure 4F). Moreover, BY4741 and Δ*Gem* cells expressing either A30P or A53T alpha-synuclein showed lower ratio of GSH/GSSG, indicating an oxidative environment in the presence of mutant alpha-synuclein (figure 4D and E).

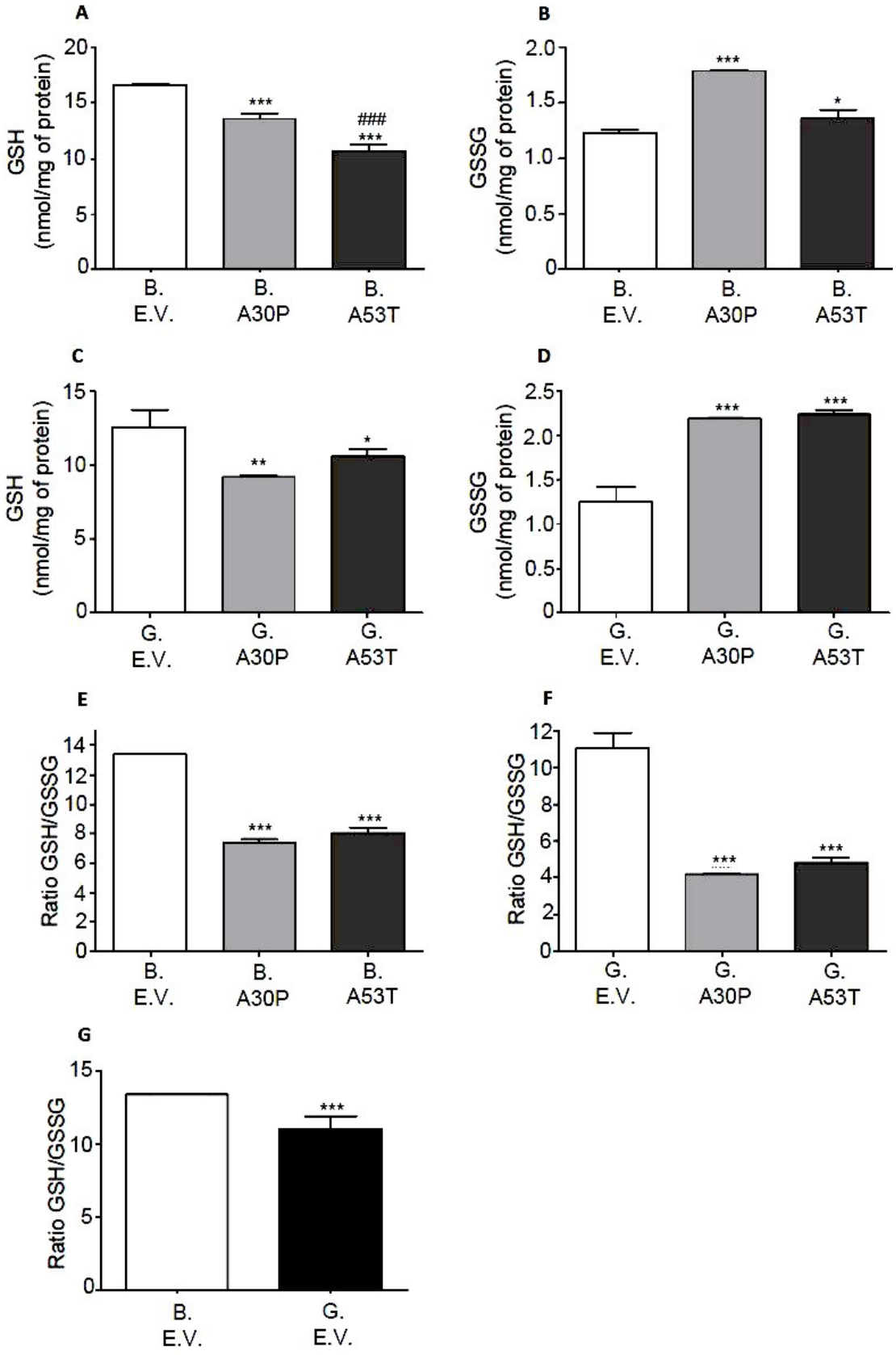

Figure 4. Quantification of GSH and GSSG levels and ratio between GSH/GSSG using HPLC-ECD. (**A**) BY4741 expressing A30P or A53T alpha-synuclein showed lower GSH levels and (**B**) higher GSSG levels compared to cells expressing E.V. (**C**) Δ*Gem*

expressing A30P or A53T alpha-synuclein showed lower levels of GSH and (**D**) higher levels of GSSG compared to cells expressing E.V. (**E**) BY4741 and (**F**) Δ*Gem* expressing A30P or A53T α-synuclein showed lower levels of GSH/GSSG ratio compared to their respective controls. (**G**) Δ*Gem* expressing E.V. showed lower GSH/ GSSG ratio compared to BY4741 expressing E.V. The values of 3 independent experiments (n=3) are expressed as absolute number ± SD. One-way ANOVA followed by Tukey post test or *t* Test were statistical test employed. *p≤ 0.05 compared with respective control. # p≤ 0.05 compared with cells expressing A30P alpha-synuclein.

Autophagy flux

To analyze autophagic flux, cells were successfully transformed with GFP-Atg8 (39 kDa) as shown in the blotting in Figure 5A. Since GFP is resistant to degradation by vacuoles, only Atg8 is cleaved in the yeast degradation, which leaves the GFP molecule free (27 kDa). The ratio between free GFP and GFP-Atg8 was analyzed to measure autophagy flux. The ratio of GFP/GFP-Atg8 was higher in Δ*Gem* cells than in BY4741, suggesting that autophagic flux is increased in these cells (Figure 5D). In the presence of both mutant alpha-synuclein, the ratio between GFP/GFP-Atg8 in the BY4741 was higher than in BY4741 expressing E.V. (Figure 5B). In Δ*Gem* cells, the ratio between GFP/GFP-Atg8 was higher only in cells expressing A53T alpha-synuclein, while the ratio in cells expressing A30P alpha-synuclein did not change (Figure 5C). Decreased autophagic flux is thought to promote protein accumulation and consequently aggregation (Ritz et al., 2016). Therefore, these data indicate the absence of Gem by itself increases autophagy in yeasts, which consequently could prevent the formation of protein aggregates.

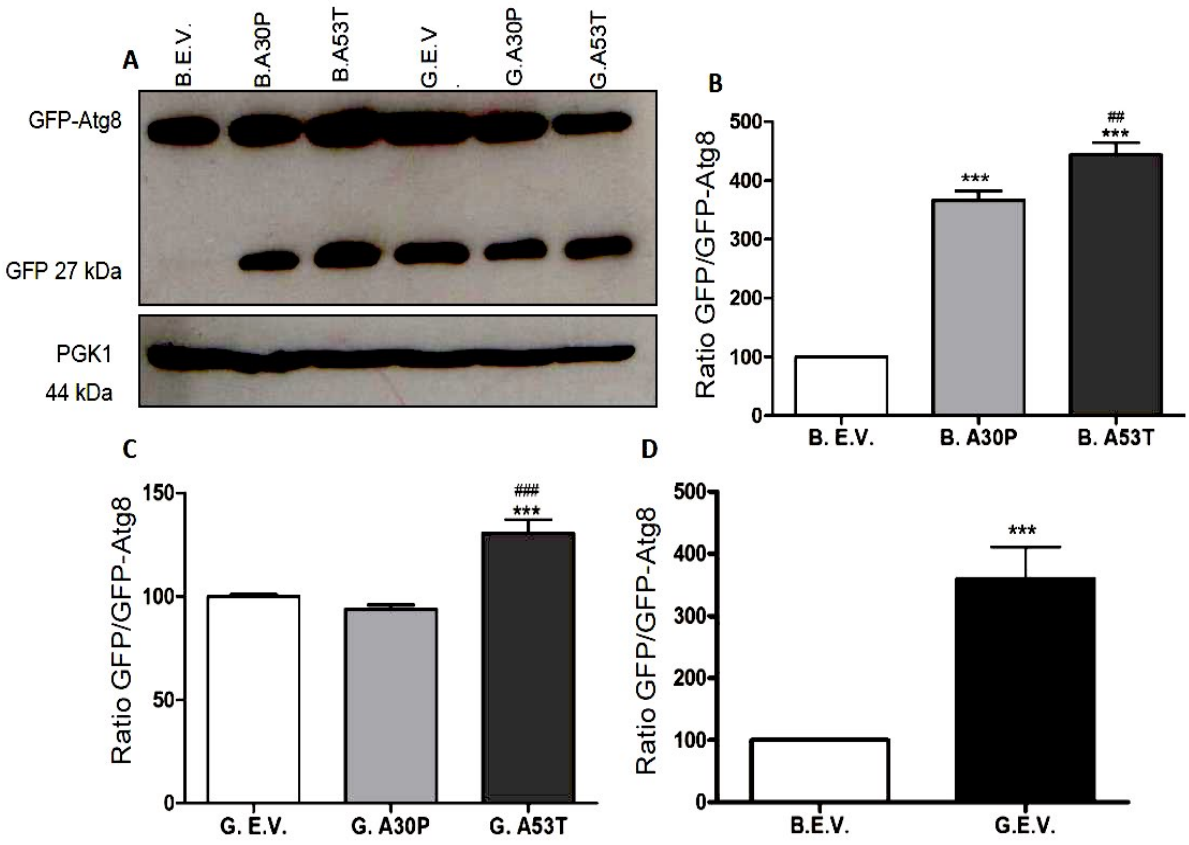

Figure 5: Autophagic flux analysis through evaluation of ratio between free GFP and GFP-Atg8. (**A**) Blotting showing the expression of GFP-Atg8 (39kDa), free GFP (27kDa) and normalizer Pgk1 (44kDa). (**B**) The expression of A30P or A53T alpha-synuclein increased autophagic flux in BY4741 strain compared to its respective control. (**C**) Δ*Gem* cells expressing A53T showed increased autophagic flux compared to cells expressing E.V or A30P alpha-synuclein. (**D**) The deletion of *Gem* gene lead to higher autophagic flux. The values of 3 independent experiments (n=3) are expressed as percent of control ± SD. One-way ANOVA followed by Tukey post test or *t* Test were statistical test employed. *p≤ 0.05 compared with respective control. # p≤ 0.05 compared with cells expressing A30P alpha-synuclein.

UPR and ER stress analysis by evaluation of ^{S}*Hac1*/^{U}*Hac1, Pdi* and *Ero1* mRNA

The presence of mutant alpha-synuclein, altered autophagy, and mitochondrial function and can lead to ER stress and UPR activation to restore the cellular homeostasis (Redmann et al., 2016; Colla et al., 2012; Winslow et al., 2011). To further investigate ER stress and UPR activation, levels of ^{U}*Hac1* and ^{S}*Hac1*, *Pdi1*, and *Ero1* were analyzed and compared. All cells showed a spliced form of *Hac1*, except BY4741 expressing A53T alpha-synuclein, suggesting that the toxicity of A53T alpha-synuclein

toxicity impedes the restoration of homeostasis (Figure 6A). BY4741 expressing A30P alpha-synuclein showed a stronger spliced *Hac1* band, however, the ^{U}*Hac1*/^{S}*Hac1* ratio lower than cells expressing E.V. revealing that the presence of alpha-synuclein can lead to ER stress by increasing both forms of *Hac1* (Figure 6B). When comparing the control with the Δ cells, the Δ*Gem* cells showed higher ^{S}*Hac1*/^{U}*Hac1* ratio than BY4741, indicating the deletion of the *Gem* leads to stronger ER stress (Figure 6D). The Δ*Gem* cells expressing A30P or A53T alpha-synuclein showed lower ^{S}*Hac1*/^{U}*Hac1* ratio than the Δ*Gem* cells expressing E.V., revealing that the presence of mutant alpha-synuclein reduces ER stress in the absence of *Gem* (Figure 6C). The levels of *Pdi1* mRNA were increased in the BY4741 cells expressing A53T alpha-synuclein, while cells expressing A30P alpha-synuclein did show a significant difference of Pdi1 expression compared to cells expressing E.V. (Figure 6E). Δ*Gem* cells expressing both mutant alpha-synucleins showed increased expression of *Pdi1* than cells expressing E.V. (Figure 6F). Furthermore, *Ero1* levels were higher in the BY4741 and Δ*Gem* cells expressing both mutant alpha-synuclein, suggesting that *Pdi1* activity can be enhanced in these cells (Figure 6G and H). Taken together, these results suggest that the pathway to alleviate ER stress through the UPR is activated, likely promoting BY4741 cell survival in the presence of A30P alpha-synuclein or in absence of the *Gem* and in the presence of both mutant alpha-synuclein.

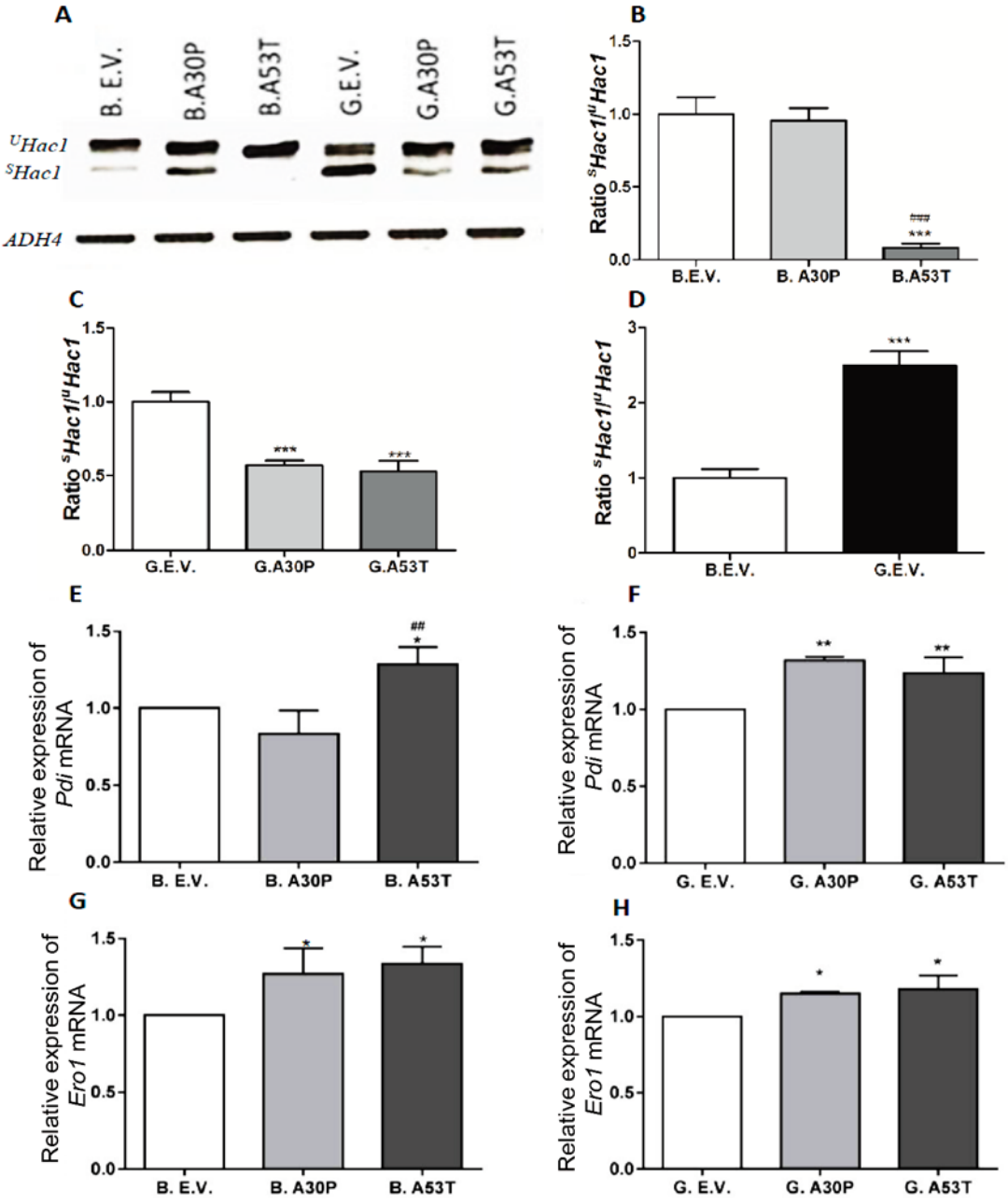

Figure 6: mRNA expression and ratio of ^{S}*Hac1* (650 bp) and ^{U}*Hac1* (splicing 450 bp) and loading control *ADH4* (1369 bp) and qRT-PCR of *Pdi1* and *Ero1*. (**A**) BY4741 and *ΔGem* cells showed *Hac1* spliced indicating ER stress, except BY4741 cells expressing A53T alpha-synuclein. (**B**) BY4741 cells expressing A53T alpha-synuclein did not show *Hac1* splicing and cells expressing A30P alpha-synuclein did not show significantly differences between ^{S}*Hac1*/ ^{U}*Hac1* ratio compared to cells expressing E.V. (**C**) *ΔGem* cells expressing A30P or A53T alpha-synuclein showed lower ^{S}*Hac1*/ ^{U}*Hac1* ratio than its respective control expressing E.V. (**D**) *ΔGem* cells expressing E.V. showed

higher $^{S}Hac1/^{U}Hac1$ ratio than BY4741 cells expressing E.V. (**E**) BY4741 expressing A53T alpha-synuclein showed higher *Pdi1* mRNA levels compared to cells expressing E.V. or A30P alpha-synuclein. (**F**) *ΔGem* cells expressing A30P or A53T alpha-synuclein showed higher *Pdi1* mRNA levels compared to cells expressing E.V. (**G**) BY4741 expressing A30P or A53T alpha-synuclein showed higher *Ero1* mRNA levels than cells expressing E.V. (**H**) *ΔGem* expressing A30P or A53T alpha-synuclein showed higher *Ero1* mRNA levels than cells expressing E.V. The values of 3 independent experiments (n=3) are expressed as absolute number ± SD. One-way ANOVA followed by Tukey post test or *t* Test were statistical test employed. *p≤ 0.05 compared with respective control. # p≤ 0.05 compared with cells expressing A30P alpha-synuclein.

DISCUSSION

Most *in vitro* studies about PD utilize mammalian cells to investigate the cellular and molecular mechanisms of alpha-synuclein toxicity. However, the role of some genes, such as Miro (the mammalian orthologue of yeast Gem) in the presence of alpha-synuclein could be better analyzed using a knockout model, which is impossible to create using mammalian cells (Guo et al., 2005). The simple humanized *S. cerevisiae* (yeast) model has been widely used to study neurodegenerative diseases and has produced useful data that have been used to clarify the mechanisms involved in the pathology of PD and other diseases (Ciaccioli et al., 2013; Franssens et al., 2013). Therefore, we used BY4741 (control) and BY4741 with *Gem* deletion (Δ*Gem*) yeasts transformed with A30P or A53T alpha-synuclein to investigate mitochondrial, ER, and autophagy dysfunction. Our findings revealed that the presence of mutant alpha-synuclein impaired the control yeast's (BY4741 expressing E.V.) growth and led to an oxidative environment, aberrant autophagy, and ER stress. The toxic effect of A53T alpha-synuclein was even stronger, forming more aggregates and leading to higher H_2O_2 levels produced by mitochondria, which corroborates with the results of other studies using yeast or other models (Chen et al., 2015; Ciaccioli et al., 2013). Our study was the first to show that the deletion of the Gem gene in yeast led to a comparable state as the presence of A53T alpha-synuclein in the control strain. However, the presence of mutant proteins attenuates the effects of *Gem* deletion, revealing that *Gem* deletion promotes cell protection against the toxic effects caused by mutant alpha-synuclein and indicates that *Gem* could play a role in mitochondrial dysfunction caused by A53T

alpha-synuclein.

It has been shown that A53T alpha-synuclein oligomerizes and forms aggregates faster and more readily than A30P or WT alpha-synuclein (Ostrerova-Golts et al., 2000; Stefanovic et al., 2015). Interesting, even it was used a known very effective protocol to break down proteins, the BY4741 cells expressing A53T alpha-synuclein showed a band in the stacking gel, which is a strong indicator of protein aggregation. Surprisingly, the Δ*Gem* did not show any indication that A53T alpha-synuclein was aggregated (Figure 1A). However, in Figure 2 both mutant proteins were detected in fraction P_{1000} in both lines, indicating the presence of oligomers and aggregates when a specific protocol to identify aggregates was used. Taken together these results suggest that A53T alpha-synuclein forms less aggregates in the Δ*Gem* cells, likely due to higher autophagy flux in comparison to control cells; the autophagy flux was even higher in Δ*Gem* cells expressing A53T alpha-synuclein as shown in Figure 5. It has been reported that autophagy inducers can decrease the amount of alpha-synuclein and prevent protein aggregation (Ebrahimi-Fakhari et al., 2013; Vilageliu and Grinberg, 2017). In Figure 5, we observed that BY4741 cells expressing mutant alpha-synuclein showed increased autophagic flux, while Δ*Gem* cells expressing A30P or A53T alpha-synuclein showed even higher autophagic flux. These set of data indicates that both groups demonstrated protective mechanisms against alpha-synuclein accumulation, nevertheless, this mechanism seemed more effective in Δ*Gem* cells suggesting that *Gem* deletion delays A53T alpha-synuclein aggregation.

Next, we observed that the deletion of *Gem* led to decreased cell viability compared to control cells, revealing that *Gem* is essential for the normal yeast cell cycle. As expected, the presence of both mutant proteins in BY4741 decreased control cell viability (Figure 1), which is in agreement with previous studies that have shown the toxic effects of A30P and A53T alpha-synuclein on yeast growth (Ciaccioli et al., 2013; Fruhmann et al., 2017). Intriguingly, we found that expression of both mutant alpha-synuclein types in Δ*Gem* did not affect cell viability, suggesting that the deletion of *Gem* protects cells against alpha-synuclein toxicity. Furthermore, the growth curve analysis demonstrated that control cells expressing A53T alpha-synuclein had even more difficulty to grow (Figure 1D and D1), indicating that these cells were more

vulnerable to A53T alpha-synuclein toxicity, which could impair the activation of mechanisms that prevent apoptosis, conferring susceptibility of cells to death, and impede the restoration of homeostasis. Indeed, BY4741 expressing A53T alpha-synuclein cells were more susceptible to death than cells expressing E.V. or A30P alpha-synuclein (Supplemental Figure 3). Moreover, UPR is considered one of the most important mechanisms to restore homeostasis when protein accumulates (Delic et al., 2012) and in figure 6A it is shown that all strains expressed ^{U}*Hac1* and ^{S}*Hac1* except the control cells expressing A53T alpha-synuclein, indicating that mechanisms to restore homeostasis were not activated through the *Hac1* pathway in these cells leaving them more prone to death. On the other hand, the folding machinery in the ER was activated as the mRNA of *Pdi* and *Ero1* expression is increased in these cells (Figure 6E and G), as well as in the control cells expressing A30P alpha-synuclein and Δ*Gem* cells expressing both mutant proteins. Curiously, both mutant alpha-synucleins were not found in the ER fraction (Figure 2). However, it is known that mitochondrial dysfunction and the production of H_2O_2 affect the ER and lead to ER stress via the cross-talk between the organelles, which is mediated by Gem, as cited previously. The activation of Pdi generates H_2O_2 that leads to an oxidative environment. Recent findings demonstrated that Pdi1 and Ero1 levels were increased in the presence of alpha-synuclein and altered autophagy leading to ER stress. However, inhibition of Pdi1 completed annihilated ER stress, decreased the levels of Ero1 and increased autophagic flux (Lehtonen et al., 2016). These data suggest that normal Pdi1 levels are crucial to prevent ER stress and to keep ER homeostasis. In fact, both groups expressing mutant alpha-synuclein had a lower ratio of GSH/GSSG, indicating an oxidative environment (Figure 4). The consumption of total GSH and generation of GSSG could also be due to higher H_2O_2 levels produced by the mitochondria. Mitochondrial dysfunction caused by the overexpression of alpha-synuclein or expression of mutated alpha-synuclein also increases ROS levels, such as H_2O_2 (Nakamura, 2013). In Figure 3 we observed that Δ*Gem* mitochondria produced more H_2O_2 than control cells. Interestingly, BY4741 expressing A53T alpha-synuclein showed higher levels of H_2O_2 produced by mitochondria being comparable with the H_2O_2 levels produced by mitochondria from Δ*Gem*, while mitochondria from Δ*Gem* expressing A53T alpha-synuclein produced

lower levels of H_2O_2, which were comparable to the H_2O_2 levels produced by mitochondria from control cells, suggesting that the deletion of the *Gem* allowed the cells to rescue basal levels of H_2O_2 production. Moreover, in the absence of Gem, the ^{U}*Hac1*/^{S}*Hac1* ratio was lower, indicating a disruption of the cross-talk between the mitochondria and ER, which could protect cells against ER stress caused by dysfunctional mitochondria.

The mechanisms behind mitochondrial dysfunction caused by alpha-synuclein are still not completely clear. Nevertheless, it has been shown that A53T alpha-synuclein damaged mitochondria more intensively by interacting with the mitochondrial membrane, generating pores, decreasing mitochondrial membrane potential, and leading to increased H_2O_2 production. Indeed, we found A53T alpha-synuclein in mitochondrial fractions, suggesting that the mutant protein could interact with mitochondrial membrane in both strains (Figure 2). However, A30P alpha-synuclein did not interact with the mitochondrial membrane and its toxicity was more related to proteasome inhibition (Smith et al., 2005). In fact, A30P alpha-synuclein did not change mitochondrial production of H_2O_2 in control or Δ*Gem* cells, which may be because the protein does not directly damage the mitochondrial membrane. Taken together, these results suggest that mutant alpha-synuclein toxicity involves mitochondrial damage, ER stress and susceptibility to apoptosis, and that the deletion of the Gem gene could prevent alpha-synuclein toxicity related to mitochondrial and autophagy dysfunction events.

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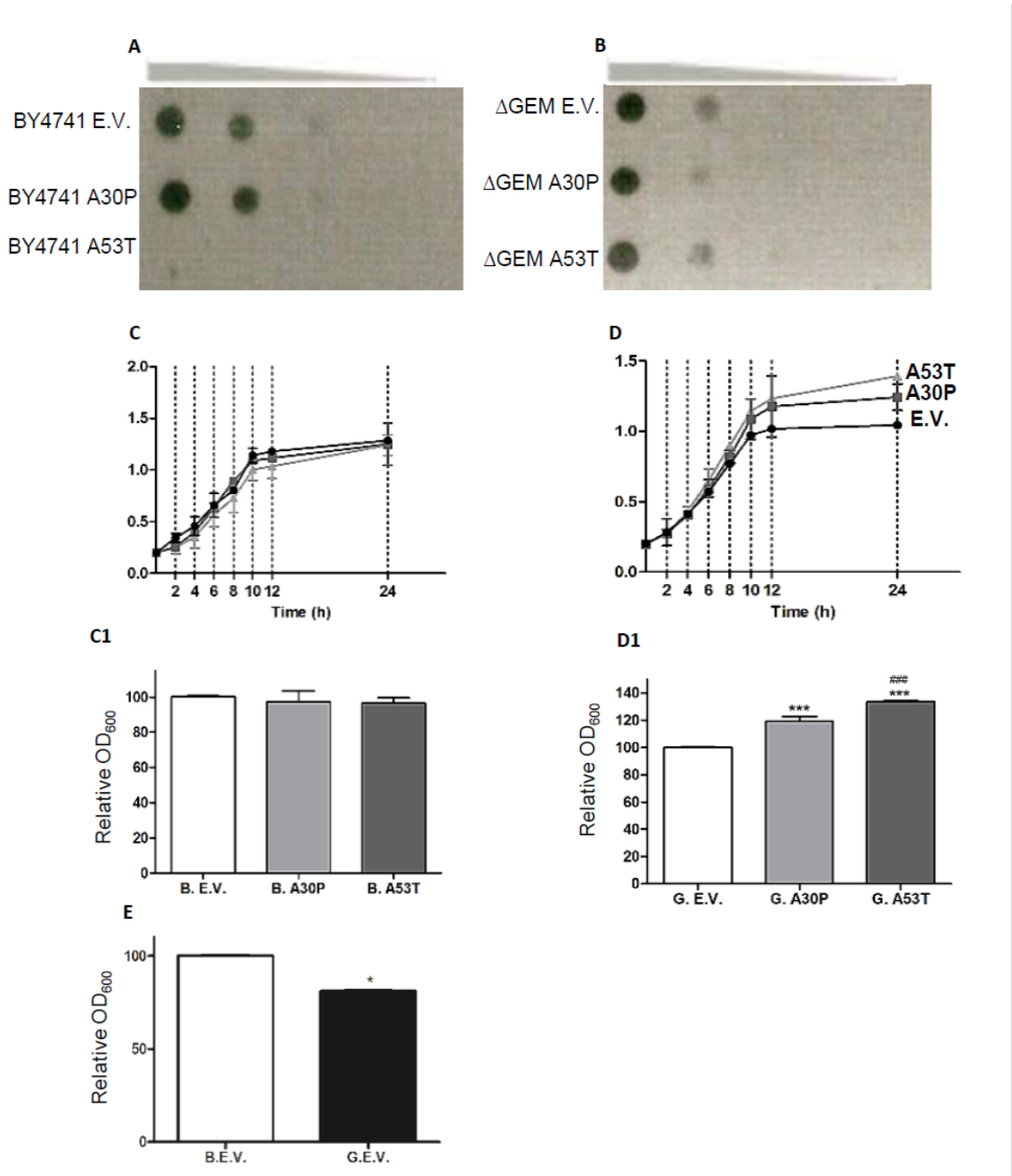
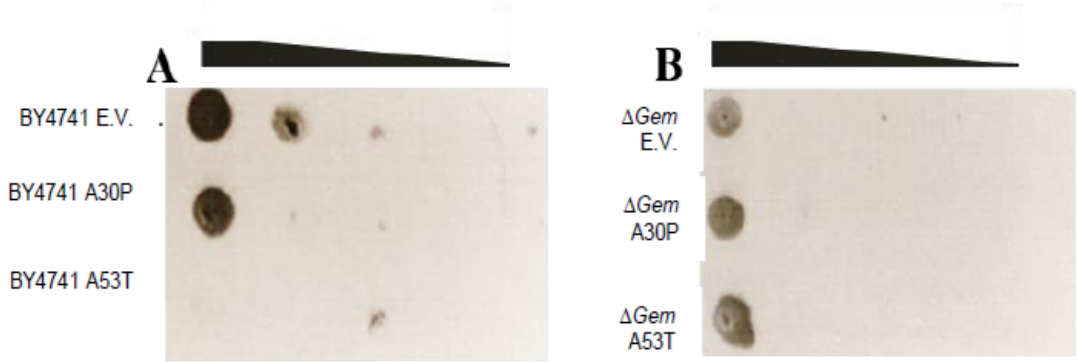
Supplementary information for chapter 6:

Supplementary figures:

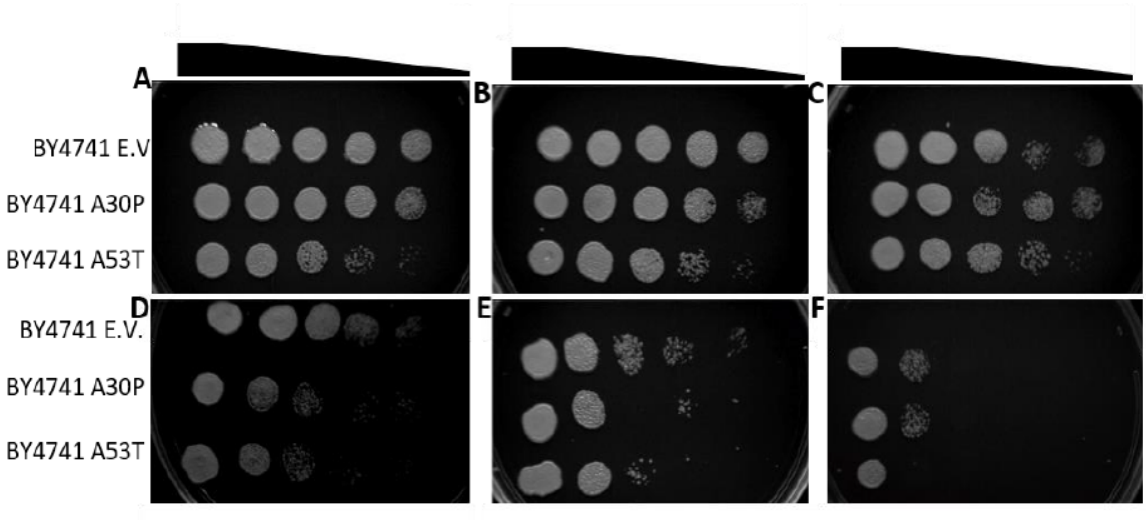


Supplemental Figure 1: Viability of cell strains expressing A30P or A53T α-synuclein cultured in SG medium. A and B: dilution series from a concentration at 1.0 OD, diluted at 1/10; 1/100; 1/1000; 1/10000 cultured for 4 days. C and D: growth curve from a concentration at 0.2 OD measured every 2h. C1, D1 and E: Quantification of growth curve at 24h. A53T alpha-synuclein impaired normal growth of BY4741 (A). Absence of *Gem* impaired normal growth of yeasts, compared to BY4741 (E). A30P and A53T alpha-synuclein ameliorate Δ*Gem* growth (D and D1). The values of 3 independent experiments (n=3) are expressed as percentage of control ± SD. One-way ANOVA followed by Tukey post test or *t* Test were statistical test employed. *p≤ 0.05 compared

with respective control. # p≤ 0.05 compared with cells expressing A30P alpha-synuclein.



Supplemental Figure 2: Viability of BY4741 or Δ*Gem* expressing A30P or A53T α-synuclein cultured in SGE. A and B: dilution series from a concentration at 1.0 OD, diluted at 1/10; 1/100; 1/1000; 1/10000 cultured for 8 days. Both strains showed difficulties to grow in SGE medium.



Supplemental Figure 3: Sensitivity assay of BY4741 expressing A30P or A53T α-synuclein, cultured in SD medium. Cell lines were exposured to H_2O_2 at 0mM (A); 0.5mM (B); 1.0mM (C); 1.5mM (D); 2.0mM (E); 3mM (F); for 8 days. Yeasts were plated in series dilution at 1/10; 1/100; 1/1000; 1/10000 from 1.0 O.D. A53T alpha-synuclein impaired yeasts growth. Concentrations of H_2O_2 upon 1.0mM impaired normal growth in all cell lines.

CHAPTER 7

SUMMARY AND CONCLUSIONS

Recent reports have shown that alpha-synuclein leads to abnormal mitochondrial dynamics, aberrant autophagy and increased ROS worsening mitochondrial dysfunction (Giordano et al., 2014; Redmann et al., 2016). Especially mutated alpha-synuclein or overexpression of the wild type (WT) protein overloads lysosome and endoplasmic reticulum (ER). Consequently, degradation, in general, is impaired in the cell, including degradation of alpha-synuclein and mitochondria, favoring protein aggregates formation and mitochondrial dysfunction (Freeman et al., 2013; Sarkar et al., 2016; Winslow and Rubinsztein, 2011). Moreover, abnormal mitochondrial functioning and overloaded endoplasmic reticulum cause ER stress and increased oxidative stress in turn worsens mitochondrial dysfunction (Grimm, 2012; Sarkar et al., 2016). Together, these events create a vicious cycle leading to cell death. Recent studies, focusing on unveiling mechanisms involved in all these processes, demonstrated that mitochondria are the first to be affected by alpha-synuclein and that mitochondrial dysfunction subsequently leads to stress of organelles such as lysosome and endoplasmic reticulum (Arduino et al., 2013; Chen et al., 2015; Grimm, 2012; Krols et al., 2016). The mechanisms behind mitochondrial dysfunction caused by alpha-synuclein are still unclear and the major focus of alpha-synuclein toxicity studies in PD.

It is known that mitochondrial function is dependent of perfect maintenance of mitochondrial dynamics, which consists basically in equilibrated anterograde and retrograde trafficking and anchoring mitochondria in sites with a high demand for energy (Cieri et al., 2017; Sheng, 2014). Anterograde transport is crucial to the maintenance of synapses, whereas, retrograde transport is crucial for renewing mitochondria since mitochondrial biogenesis occurs preferentially at the soma (Sheng, 2014). Accordingly, studies have shown that impaired mitochondrial trafficking is involved in the earliest stages of PD pathogenesis (Bose and Beal, 2016).

In the present studies, we have used different cell models and aimed to investigate the mechanisms underlying mitochondrial dysfunction as a consequence to rotenone exposure or caused by the overexpression of different types of alpha-synuclein. Moreover, we aimed to investigate alterations in autophagy and endoplasmic reticulum stress, which have been shown to be worsened by or a consequence of mitochondrial dysfunction (Arduino et al., 2013; Grimm, 2012; Krols et al., 2016; Su

and Qi, 2013).

In **chapter 1**, we have given a short overview on factors that contribute to PD susceptibility, focused on population aging around the world, and described therapies available for patients and the impact on families and society in concern of the costs of the treatments for this disease. Furthermore, we discuss clinical symptoms during the development of the disease and the link between aging and the development of the disease, since aging appears to be the main risk for PD. Moreover, we have discussed major genetic factors determining the susceptibility to PD and finally explained how different models are crucial to reveal the mechanisms behind the PD pathology in different scenarios.

In **chapter 2**, we provided an extensive review about the cellular and molecular characteristics of PD, on similarities between the aging process and PD development, covering the unique metabolism of dopaminergic neurons, but focused, in particular, on alpha-synuclein including its normal function and its pathogenic effects on intracellular trafficking and cellular stress. We addressed how overexpression and mutations in alpha-synuclein lead to dysfunction of mitochondria, endoplasmic reticulum and autophagy.

In **chapter 3**, we described our experiments with cultured DA neurons isolated from substantia nigra of neonatal rats. We treated these cells with low doses of rotenone and analyzed the motor proteins expression and total mitochondrial trafficking. We have observed that especially the expression of anterograde motor KIF1B was increased after rotenone exposure at 0.5nM (the highest concentration employed in this study). The analysis of the total mitochondrial trafficking using mitotracker green revealed a decrease in mitochondrial mobility after rotenone exposure at 0.1or 0.5nM. The number of mitochondria labeled by mitotracker orange did not change among the groups, revealing the treatment with rotenone did not cause mitochondrial membrane potential alterations. As cited above, both directions of mitochondrial trafficking are crucial for keeping mitochondrial dynamics and, therefore, specific impairment in one of these directions could lead to specific mitochondrial damage. It has been shown that impairment in the mitochondrial anterograde trafficking affects synapse formation and dendrites growth, while impairment of retrograde mitochondrial trafficking affects

mitochondrial biogenesis (Van Laar and Berman, 2009). However, due to the complexity and variability of the primary cell culture network, it was impossible to analyze the anterograde or retrograde mitochondrial trafficking. Therefore, the employment of other cell models was needed to investigate how the impairment of mitochondrial trafficking could lead to mitochondrial damage.

In **chapter 4**, we reported the culture of human SH-SY5Y cells (neuroblastoma) derived neurons transgenic for WT, A30P or A53T alpha-synuclein. In contrast to our primary cell model, SH-SY5Y neurons allowed assessment of the direction of mitochondrial trafficking, providing the possibility to determine how specific impairment of the anterograde or retrograde direction of trafficking affects mitochondrial function. We assessed the total and specific directions of intracellular trafficking in the presence of WT or mutated alpha-synuclein in differentiated SH-SY5Y cells and the consequences on mitochondrial dynamics. Although most cases of PD are sporadic, only about 10% of cases have defined genetic causes(Kalinderi et al., 2016). Cellular models expressing mutated alpha-synuclein and/or exposed to neurotoxins that damage mitochondria are widely used to study time and conditions relevant for the development of the PD phenotype. We observed that mitochondrial retrograde trafficking was impaired in neurons expressing A53T alpha-synuclein after 6 DIV, however, after 8 DIV both directions of trafficking were impaired in these neurons, while neurons expressing WT or A30P alpha-synuclein did not show any differences in mitochondrial mobility. The expression of A53T alpha-synuclein also led to mitochondrial distribution and connectivity impairment, indicating the importance of mitochondrial trafficking for keeping mitochondrial localization and network properly connected. Interesting, neurons expressing WT or mutant alpha-synuclein showed higher levels of ROS than control neurons, with the highest ROS levels presented in neurons that expressed A53T alpha-synuclein, indicating that all types of alpha-synuclein expressed lead to oxidative stress, mainly caused by mitochondrial dysfunction. Since it is known that WT, A30P and A53T alpha-synuclein lead to PD and A53T oligomerizes faster than other types of alpha-synuclein, likely with longer SH-SY5Y culture or addition of neurotoxin, we might have observed the same alterations in mitochondrial trafficking and dynamics in cells expressing WT or A30P alpha-

synuclein. It is known that disrupted microtubules can lead to impairment of general axonal trafficking and that alpha-synuclein can play a role in microtubules disassembly. In order to recover mitochondrial trafficking, we treated neurons with the neuropeptide NAP, which facilitates microtubule assembly. We have used NAP at a concentration that restores mitochondrial trafficking and consequently, the levels of ROS were normalized in neurons expressing any type of alpha-synuclein. Moreover, mitochondrial connectivity, distribution and morphology were recovered in NAP treated neurons that expressed A53T alpha-synuclein, revealing that disturbed trafficking can be the key to the cascade of cellular alterations in PD.

In order to investigate alterations in mitochondrial trafficking and dynamics in human neurons, **in chapter 5**, we generated hiPSCs from 2 patients with PD: one having a SNCA3 mutation, that led to overexpression of WT alpha-synuclein and one with a point-mutation in the alpha-synuclein gene, A53T. Cells were differentiated and purified into neurons and cultured for 90 days. It was observed that mitochondrial trafficking was decreased in both lines confirming that disrupted mitochondrial trafficking is an important characteristic of PD pathology. Furthermore, we found mitochondria with abnormal morphology accumulated in the soma and indications that the organelles were fragmented. This is the first study showing impaired mitochondrial dynamics in a long-term neuronal culture of PD patients containing SNCA3 or A53T mutations. Culturing neurons for 90 days is a challenge, especially neurons containing the SNCA3 mutation. It has been demonstrated that the SNCA3 mutation impairs the differentiation and maturation of neurons, especially DA neurons. Moreover, neural precursor cells from SNCA3 patient showed colocalization of alpha-synuclein and mitochondria, consequently higher levels of ROS and higher vulnerability of apoptosis than control (Flierl et al., 2014) (Oliveira et al., 2015). Together with our results, these findings show that alpha-synuclein targets mitochondria early in PD and that disrupted mitochondrial trafficking is linked to the organelle dysfunction in neurons from PD patients.

How alpha-synuclein triggers mitochondrial dysfunction is still unclear. It is thought that proteins related to mitochondrial dynamics are involved in the mechanisms that lead to abnormal mitochondrial functioning in the presence of alpha-synuclein.

Several studies have shown that especially Miro plays an important role in all processes involved in mitochondrial dynamics (Devine et al., 2016). The selective knock-out of proteins/genes has been used in many cell models to clarify their role in cellular events that contribute to neurodegeneration. However, the viability of polarized cells like neurons, are totally dependent of intracellular trafficking. Therefore, in **chapter 6,** we have used a yeast model for investigating the relation of alpha-synuclein toxicity and Miro on mitochondrial and also autophagy and ER dysfunction. As expected, we have found that A30P alpha-synuclein toxicity differs from A53T alpha-synuclein toxicity which is more severe in the last one. Cells expressing A53T alpha-synuclein showed an indication of protein aggregate and changes in mitochondrial and autophagy functioning that could be prevented or reduced by the absence of Gem (the yeast orthologue of Miro in mammalian). These data indicate that understanding the mechanisms which lead to alpha-synuclein aggregation and toxicity involve specific signalization of genes related to mitochondrial dynamics. Yeasts Δ*Gem* (knock-out of Gem), showed decreased cellular viability. Curiously, Δ*Gem* cells expressing mutant alpha-synuclein showed ameliorated viability and also increased autophagic flux, decreased levels of hydrogen peroxide and decreased endoplasmic reticulum stress. Together these data reveal that Miro contributes to alpha-synuclein toxicity related to oxidative stress and autophagic flux.

It is well known that mitochondrial dysfunction can lead to degeneration of DA neurons. However, the mechanisms that lead to mitochondrial dysfunction are still unclear. In this thesis, we showed that not only alteration but, specifically the decrease of mitochondrial trafficking is a common event in 3 different experimental cell models for PD, indicating that disrupted mitochondrial mobility can contribute to cause or worsen mitochondrial dysfunction in a sporadic or familial model of PD. Furthermore, we found that Miro, which is responsible for maintaining a normal mitochondrial distribution by controlling normal microtubules dynamics, is involved in the mechanisms underlying mutant alpha-synuclein toxicity involving protein aggregation, mitochondrial and autophagy dysfunction. Its absence can prevent oxidative stress caused by mutant alpha-synuclein.

As indicated previously, alpha-synuclein oligomers can lead to disrupted

mitochondrial trafficking by interaction with tau, promoting microtubule disassembly or also by interaction with motor proteins related to mitochondrial anterograde or retrograde transport (Prots et al., 2013). The abnormal distribution of mitochondria or the absence of Miro, both have been reported to impair microtubules dynamics (Iijima-Ando et al., 2012; Morlino et al., 2014). However, interactions between alpha-synuclein and the motor protein Miro are unknown. Interestingly, we found that recovering microtubule assembly with NAP and removing Miro, which is indirectly involved in microtubules growth and dynamics, could recover mitochondrial function and prevent mitochondrial dysfunction, respectively, caused by mutant alpha-synuclein.

It has been shown that non-functional Miro or impaired Miro turnover can lead to abnormal mitochondrial morphology and mitophagy (Birsa et al., 2014; Fransson et al., 2006; Kazlauskaite et al., 2014; MacAskill and Kittler, 2010). Our findings using differentiated SH-SY5Y and neurons from patients with PD showed that the disruption of the mitochondrial trafficking occurs concomitant with the appearance of fragmented mitochondria. In addition, differentiated SH-SY5Y and yeast expressing A53T alpha-synuclein showed higher levels of ROS, indicating mitochondrial dysfunction. Once again, these changes were recovered after NAP treatment or depletion of Miro. Intriguingly, Arduíno and colleagues (Arduino et al., 2013), showed that in an animal model for PD, accumulation of dysfunctional mitochondria triggers microtubule disassembly. Taken together, these findings indicate that alpha-synuclein disrupts microtubules indirectly by damaging mitochondria or maybe via an interaction with Miro. Whether alpha-synuclein interacts directly with Miro and disturbs Miro dynamics or with proteins related to Miro dynamics remains elusive.

Autophagy pathology is associated with protein aggregation and is considered a hallmark of PD. In our model using yeasts, expression of A53T alpha-synuclein led to the highest autophagic flux. Interesting, the absence of Miro in yeasts expressing A53T alpha-synuclein led to even higher autophagic flux indicating Miro depletion prevented protein aggregation by increasing protein degradation. Curiously, it has been demonstrated that dysfunctional mitochondria can decrease autophagy flux in PD model (Arduino et al., 2013). These data strongly suggest that absence of Miro can prevent mitochondrial damage caused by mutant alpha-synuclein and consequently also prevent

autophagic flux decrease.

As commented in chapter 2 and 6, mutant alpha-synuclein is commonly found in the ER of mammalian models for PD. In these models, alpha-synuclein disturbs the organelle homeostasis leading to ER stress and mitochondrial dysfunction in a vicious cycle of stress due to the cross-talking between the organelles. However, yeast cells did not show alpha-synuclein staining in the ER, suggesting the stress observed is not caused directly by the presence of the protein inside the organelle, but likely by other cellular damage caused by alpha-synuclein.

Together, our models revealed that mitochondrial dysfunction caused by alpha-synuclein toxicity involves a disruption in intracellular trafficking, preferentially first in mitochondrial retrograde trafficking. Besides that, alpha-synuclein toxicity is dependent of Miro (Gem) a protein related to mitochondrial trafficking and dynamics, demonstrating that understanding the mechanisms behind the impairment of intracellular trafficking could be key to unveil PD cellular pathogenesis.

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CHAPTER 8

DUTCH SUMMARY

SAMENVATTING

Recente publicaties hebben laten zien dat alpha-synucleine een rol kan spelen in het ontstaan van het disfunctioneren van mitochondriën al dan niet gepaard met afwijkingen in autofagie; met name als er sprake is van over-expressie van alpha-synucleine of van gemuteerd alpha-synucleine raken de lysosomen en het endoplasmatisch reticulum in zenuwcellen overbelast. De afbraak van alpha-synucleine en het opruimen van beschadigde mitochondriën raakt verstoord, met eiwit-aggregatie en verdere mitochondriale dysfunctie als gevolg. Dit alles leidt weer tot stress van het endoplasmatisch reticulum en toegenomen oxidatieve stress in de cel, een vicieuze cirkel van verslechtering van mitochondriale functie met uiteindelijk celdood als gevolg. Het exacte mechanisme achter de toxische interactie tussen alpha-synucleine en mitochondriën is nog grotendeels onbekend en vormt het focus van tal van alpha-synucleine toxiciteitsstudies in het kader van de ziekte van Parkinson. Een van de ideeën is dat een overmaat aan (al dan niet gemuteerd) alpha-synucleine mogelijk primair de mobiliteit van mitochondriën verstoort. Een perfecte balans in anterograad en retrograad transport van mitochondriën is essentieel voor normale mitochondriale functie, ervoor zorgend dat mitochondriën tijdig op plaatsen met een hoge energievraag (o.a. synapsen) ankeren en weer vernieuwd kunnen worden in het mitochondriaal biogenesis proces dat voornamelijk gelokaliseerd is in het soma.

Met behulp van een 4-tal verschillende celmodellen, i.e. a. primaire dopaminerge neuronen blootgesteld *in-vitro* aan rotenone, b. alpha-synucleine-getransfecteerde SH-SY5Y cellen, c. humane iPS cellen afkomstig van Parkinson patiënten met een alpha-synucleine gen mutatie en tenslotte d. gistcellen, hebben wij onderzoek verricht naar de vraag wat de mechanismes zijn achter de toxische effecten van alpha-synucleine op mitochondriale functie en de consequenties van de resulterende cel stress op autofagie.

In het introductie hoofdstuk, **hoofdstuk 1**, hebben wij, na een beschrijving van de algemene aspecten van de ziekte van Parkinson, een kort overzicht gegeven van de omgevingsfactoren die leiden tot een verhoogd risico op de ziekte van Parkinson en de sociale en maatschappelijke consequenties van de toenemende veroudering (in feite de belangrijkste risicofactor voor het krijgen van de ziekten van Parkinson) van de wereldbevolking voor de toename in het aantal Parkinson patiënten. Daarnaast

bediscussieerden wij kort de belangrijkste genetische risicofactoren voor “Parkinson” en laten tenslotte zien welke verschillende experimentele modellen er voor handen zijn om Parkinson cel-pathogenese te onderzoeken.

Vervolgens hebben wij in een uitgebreid review artikel, weergegeven in **hoofdstuk 2**, een zo compleet mogelijk overzicht gegeven van de huidige kennis, begrip en hypotheses over de normale functie van alpha-synucleine en zijn pathogene rol, met name in geval van alpha-synucleine over-expressie en gemuteerd alpha-synucleine, in intracellulair transport en het ontstaan van cellulaire stress.

De eerste experimenten, die wij hebben beschreven in **hoofdstuk 3**, werden uitgevoerd met gekweekte primaire dopaminerge neuronen geïsoleerd uit de substantia nigra van neonate ratten. Wij hebben die cellen met lage doses van het “Parkinson-gif” rotenone behandeld en de consequenties daarvan bestudeerd voor het mitochondriaal transport en de expressie van daarbij betrokken motor proteïnes. Hoewel het niet mogelijk bleek in dit celmodel om onderscheid te maken tussen anterograad en retrograad mitochondriaal transport, ontdekten wij met behulp van mitotracker green een algehele afname in mitochondriale mobiliteit na het blootstellen van de cellen aan 0.1 of 0.5 M rotenone; deze doses leken geen significante verstoringen te induceren in de mitochondriale membraan potentiaal en het aantal functionele mitochondriën bleef hetzelfde. De gebruikte doses rotenone hadden nauwelijks of geen invloed op de expressie van relevante motor proteïnes; slechts een significante toename in het anterograde motor proteïne KIF1B onder invloed van rotenone kon worden vastgesteld. Of die verhoogde concentratie van KIF1B van betekenis was voor de geobserveerde vermindering in mitochondriale mobiliteit kon niet worden vastgesteld.

In de experimenten beschreven in **hoofdstuk 4** hebben wij gebruikt gemaakt van uit SH-SY5Y (humane blastoma) cellen verkregen neuronale cellen, waarin, met behulp van gentransfectie, gemuteerd alpha-synucleine (mutaties A30P en A53T) of extra wild-type alpha-synucleine tot expressie was gebracht. In dit celmodel voor de ziekte van Parkinson konden wij, in tegenstelling tot de primaire neuronen in hoofdstuk 3, de consequenties van afwijkende alpha-synucleine expressie voor anterograad en retrograad mitochondriaal transport afzonderlijk analyseren. Na 6 dagen kweek bleek dat retrograad mitochondriaal transport verstoord was in de SH-SY5Y neuronen waarin

A53T alpha-synucleine tot expressie was gebracht; na 8 dagen kweek bleken zowel retrograad als anterograad transport beschadigd. Expressie van A30P alpha-synucleine of over-expressie van wild-type alpha-synucleine (ook bij een periode van 8 dagen kweek) bleek geen effect te hebben mitochondriale mobiliteit. Het verschil in effect tussen A53T enerzijds en A30P en WT- alpha-synucleine anderzijds, kan wellicht toegeschreven worden aan de snellere oligomerisatie van A53T alpha-synucleine; langere kweek had wellicht ook tot effecten op mitochondriale mobiliteit van A30P en WT- alpha-synucleine geleid. In ieder geval werden in alle cellen met afwijkende alpha-synucleine expressie verhoogde ROS (reactive oxygen species) concentraties gevonden duidend op verhoogde oxidatieve stress ten gevolge van suboptimale mitochondriale functie. De geobserveerde verstoringen in mitochondriaal transport in de A53T neuronen zouden veroorzaakt kunnen zijn doordat de organisatie van microtubuli aangetast is. Wij hebben de A53T neuronen behandeld met het neuropeptide NAP waarvan is aangetoond dat het de correcte assemblage van microtubuli stimuleert. Inderdaad bleken we met de behandeling met NAP in staat om de verstoringen in de mitochondriale mobiliteit door A53T alpha-synucleine te niet te doen. Bovendien normaliseerde NAP behandeling de ROS concentraties in alle andere SH-SY5Y neuronen met afwijkende alpha-synucleine expressie, daarmee het cel-pathogene belang van vroege suboptimale mitochondriale mobiliteit ten gevolge van microtubuli beschadigingen bevestigend.

In **hoofdstuk 5** hebben we gebruik gemaakt van de iPS (induced pluripotent stem cell) technologie om dopaminerge neuronen te verkrijgen van 2 (familiaire) Parkinson patiënten met mutaties in het SNCA-gen (het gen coderend voor alpha-synucleine): een patiënt had een triplicatie van het SNCA gen leidend tot over-expressie van alpha-synucleine, de andere patiënt had een puntmutatie, A53T, in dit alpha-synucleine gen. Voor de allereerste keer konden wij aantonen dat in dopaminerge neuronen van 2 familiaire Parkinsonpatiënten, in vergelijking tot even lang (i.e. 90 dagen!) gekweekte dopaminerge neuronen van gezonde controles (ook via iPS technologie verkregen), de mobiliteit van mitochondriën significant verlaagd was. Naast een verstoorde mitochondriale mobiliteit vonden we, alleen in de Parkinson neuronen, afwijkende mitochondriën en gefragmenteerde celorganellen. Onze bevindingen lijken

de primaire betrokkenheid van abnormaal alpha-synucleine in humane dopaminerge cel pathogenese via een vroege verstoring van mitochondriale mobiliteit en daaraan gekoppelde normale mitochondriale functie te bevestigen.

Er is gesuggereerd dat alpha-synucleine de mitochondriale mobiliteit verstoort via zijn interactie met eiwitten die een cruciale rol spelen in de dynamiek van mitochondriën, met name het proteïne Miro. In veel neuronale celmodellen is selectieve knock-out van genen gebruikt om de rol van Miro in mitochondriaal transport te bestuderen, maar in gepolariseerde cellen is een kleine verstoring van intracellulair transport al fataal en is dus deze experimentele benadering onmogelijk. Daarom hebben wij in **hoofdstuk 6** gebruik gemaakt van een gistcelmodel om de mogelijke interactie tussen alpha-synucleine en Miro en de toxische effecten daarvan op mitochondriale functie, maar ook op autofagie en endoplasmatisch reticulum functie te onderzoeken. In die gistcellen brachten wij A30P en A53T alpha-synucleine via gentransfectie tot expressie en induceerden knock-out van het Gem (de gist ortoloog van Miro) gen om de rol van dit proteïne in alpha-synucleine toxiciteit te onderzoeken. Net als aangetoond in hoofdstuk 4, leidde A53T alpha-synucleine expressie ook in de gistcellen tot een ernstiger toxiciteit dan met A30P alpha-synucleine expressie. De gistcellen met A53T alpha-synucleine vertoonden proteïne-aggregatie en verstoringen in mitochondriale functie en het autofagie proces; al deze verschijnselen waren afwezig in A53T alpha-synucleine gistcellen waar het Gem (=Miro) gen niet meer tot expressie kwam.

De bevindingen uit ons alpha-synucleine onderzoek in de 4 celmodellen werden samengevat en uitgebreid bediscussieerd in **hoofdstuk 7**. Als samenvattende conclusie van onze studies kan gesteld worden dat de alpha-synucleine toxiciteit in dopaminerge neuronen lijkt te berusten op een primaire vroege verstoring van de normale mitochondriale functie, die secundaire consequenties heeft voor normale autofagie processen, de normale functie van het endoplasmatisch reticulum en het uiteindelijk optreden van ernstige celstress. De mitochondriale dysfunctie door alpha-synucleine lijkt te berusten op de verstoring van normale mitochondriale mobiliteit doordat alpha-synucleine de opbouw en structuur van microtubuli, waarschijnlijk via interactie met Miro, verstoord. Dit mogelijke mechanisme vindt bevestiging in de observaties dat enerzijds de afwezigheid van Miro de mitochondriale toxiciteit van alpha-synucleine

voorkomt en ook dat een behandeling met NAP, een neuropeptide dat de organisatie en structuur van microtubuli herstelt en beschermt, de toxische effecten van alpha-synucleine te niet doen.

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